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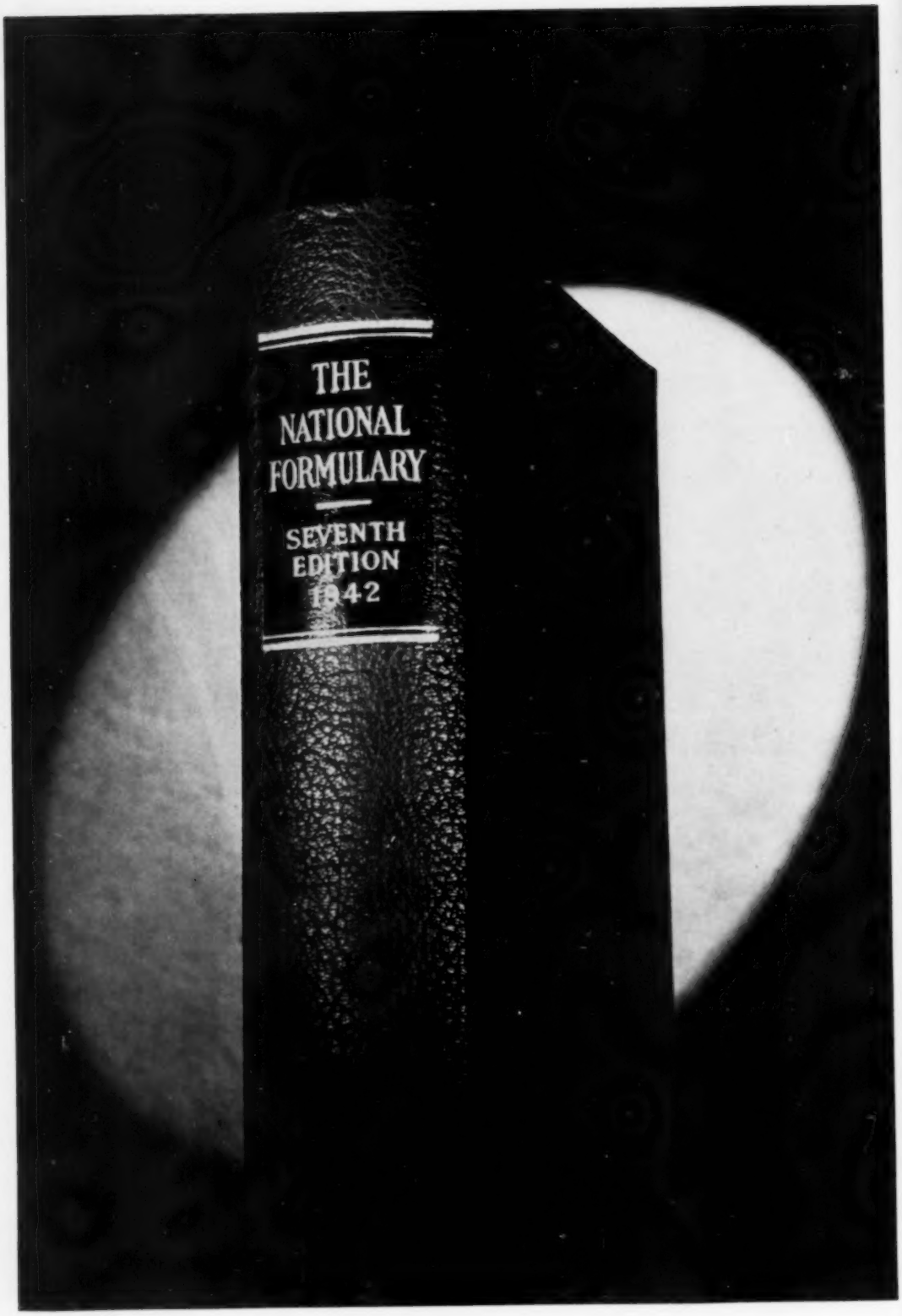
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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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On the Histological Distribution of Alkaloids in the Himalayan *Berberes**

By R. Chatterjee†‡

For the past hundred years, the members of the genus *Berberis* have interested both chemists and horticulturists. The interest of the former rests on the fact that the *Berberis* plants when subjected to chemical investigations yield a number of alkaloids (1); while the latter's interest rests on the fact that on account of their well-organized internal system, these plants offer interesting studies in hybridization (2, 3).

Although the plants of the Himalayan *Berberes* may differ in their external characteristics, they may, nevertheless, be recognized (*a*) by the thorn grouping in the joints of the stem, (*b*) by their more or less spinous toothed leaves, (*c*) by the yellow color of the scraped bark and stem, (*d*) by their yellow flowers.

The leaves of the species under consideration, *viz.*, *B. aristata* DC., *B. insignis* Hook. f., *B. lycium* Royle, *B. nepalensis*, Spreng, *B. Wallichiana* DC., and *B. Wallichiana* DC. var. *latifolia*, externally exhibit great variations in various features, *e. g.*, shape, size, etc. (4). *B. aristata* has nearly evergreen leaves, entirely oblong; the leaves of *B. lycium* are subsessile, subpersistent lanceolate, whereas those of *B. Wallichiana* DC. are oblong lanceolate and of *B. insignis*, linear lanceolate. The leaves of *B. nepalensis* are lanceolate, palmately 3 to 5 nerved. The leaves of the remaining *Berberes* in question are entirely different from the leaves of those above. *B. vulgaris* has obovate, spinulosely serrate leaves, *B. umbellata*, obovate, sparingly serrulate, whereas *B. Wallichiana* DC. var. *latifolia* has broadly obovate leaves. The leaves of the Himalayan *Berberes* are more or less deciduous, but those of *B. nepalensis* are persistent.

The external forms of the *Berberes* plants are extremely variable. *B. lycium* which grows in dry hot places of the Himalayas, at an altitude of 3000 to 9000 ft. and which is usually an erect rigid shrub, 6 to 8 ft. high, is found sometimes to take the form of a tree 10 to 12 ft. high. *B. aristata* and *B. vulgaris* are highly variable in height and shape with respect to tne altitude and climate of the place. *B. nepalensis* which is endemic to an elevation of 4000 to 7000 ft., growing in cooler and moist places, is found to attain a good height of about 15 to 20 ft., with a girth of 6 in. in diameter. In shady places, the very same species grows as a slender plant, generally without branches, whereas in stony places its growth is stunted to about 3 to 5 ft. off the ground.

The Himalayan *Berberes*, although varying extremely in their external features, show great resemblances in their anatomical characters. The stems of all of them contain single layers of epidermal cells with moderately thickened and cutinized outer cell walls. The hypodermis and the cortex are composed of parenchymatous cells with

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‡ The author is deeply grateful to Prof. A. Das-Gupta of Bangabasi College, Calcutta, whose efforts on botanical matters have made this study possible.

isolated strands of sclerenchyma in *B. nepalensis*, *B. Wallichiana* DC. and *B. insignis*, while the stems of *B. umbellata*, *B. Wallichiana*, DC. var. *latifolia*, *B. lycium* and *B. aristata* have a band of sclerenchyma. The cortical region is usually 8 to 10 layers thick. In mature stems, the whole of the cortex is replaced by rectangular cells of deep-seated phellogen with isolated strands or bands of sclerenchyma. In all the species, vascular bundles are surrounded by an undulated continuous sclerenchymatous pericycle and are arranged in the form of a ring varying in number from 16 to 28. They are radially separated by medullary rays which are usually 4 to 6 rows wide. In the outer part of the phloem region, just by the side of the pericycle are bast fibers which contain sclerenchymatous tissues and are separated from the inner soft bast, usually by a chain of lignified cells. The xylem in each case consists of vessels with large lumina, wood fibers of sclerenchyma and several wood parenchyma. The pith in the young stems of all the species is wide and circular in outline. It consists of polygonal, thin-walled parenchymatous cells, with scattered deposits of cutin, and has small intercellular spaces. In old stems, the pith is altogether replaced by xylem plates.

The inner structures of the leaves are similar too. The transverse sections of the leaves through the mid-rib are all arched, the lower outline being more curved than the upper one. The leaves have several layers of loosely arranged spongy tissues, a single layer of cutinized epidermis and a layer of palisade sclerenchyma with narrow secondary palisade cells, as in *B. Wallichiana* DC., and *B. umbellata* Wall. The vascular bundles are arranged in a semicircular disk and are surrounded by a sclerenchymatous sheath of pericycle. They vary from 3 to 7 in number. The hard bast of the phloem is sclerenchymatous and highly thickened. The hypodermis in all the members consists of thin-walled parenchymatous cells which are 4 to 8 layers in thickness.

In the roots, there is always a single-layered epiblema with cutinized cell walls. In old roots the cortical cells are replaced by cork-cells which extend from periderm to pericycle with sclerenchyma bands or strands as are present in the younger roots. The vascular bundles are enclosed in a wavy sclerenchymatous pericycle, whereas the phloem and xylem are arranged along different radii of the same circle. The central portion is occupied by loosely arranged parenchymatous cells of the pith. In older roots, however, the pith is replaced by xylem plates; and strands of medullary rays, made up of radially elongated cells, 4 to 6 rows wide are found running from the center to the pericycle. Several layers of fascicular cambium form a belt; in the outer region of the belt lies the phloem, while the xylem is within it. The hard bast, wood fibers of the xylem and pericycle of the roots, are all composed of sclerenchymatous tissues; isolated strands or bands of sclerenchyma being always noticed in the cortical region of the roots, both old and new.

The author, in his chemical investigations on the alkaloids of the Himalayan *Berberes* (5), was interested to find that the alkaloids occur in the maximum amount in organs of the plant which usually grow in the absence of light, *i. e.*, roots, and analyses of old tissues revealed that they possessed a high concentration of alkaloids, while young growing shoots contained very low percentages. These findings of the author, tally with those of Cromwell (6), who studied the origin and function of berberine in *B. Darwinii*, both biochemically and histologically.

The present microscopical observations of the stems of the *Berberes* reveal a typical dicotyledenous structure and unify most of Cromwell's findings. In the roots, young and old, main and fibrous, alkaloids are most abundant in a ring of parenchymatous tissues around the vascular bundles, in the sclerenchymatous pericycle and in the hard bast of the phloem. The xylem elements and cells of the cortex also show the presence of alkaloids. In the stems the distribution is more or less similar to that of the roots; the medullary rays, the outermost regions of the xylem and its cell walls all contain alkaloids. In the leaves, the sclerenchymatous hypodermis and the pericycle, the xylem of the vascular bundles in the mid-rib and the veins passing through the mesophyll show the presence of alkaloids. It was observed that in all cases the phloem in the roots, stems and the leaves was free from alkaloids.

On examining the distribution of the alkaloids in the various tissues, the fact was revealed that the Himalayan *Berberes* possess a deep-seated phellogen, and as such a large cortical area, and that alkaloids occur in the cortical tissues. The alkaloids are also distributed in tissues which are known chiefly to perform mechanical functions, *e. g.*, the sclerenchyma. But they are stored mainly in dead and other cells which take no part in metabolic activities and so are not drawn at all into metabolic changes It has been found that the accumulation of alkaloids takes place in specified tissues from year to year. A biochemical study of the variation and function of alkaloids in *B. nepalensis* and some other species of the Himalayan *Berberis* plants has been made with respect to the seasonal variations of the total alkaloid contents, the effect of feeding the plants with different chemical fertilizers and the withdrawal of elements essential to the growth and life of the plants. The results obtained in some cases show a connection between the utilization of protein in the growth of the plants and the syntheses and storing of alkaloids (7).

The histological distribution of alkaloids, therefore, leads to the deduction that the alkaloids in the Himalayan *Berberes* are merely stored as waste products, a view put forward on biochemical grounds by Pictet (8), and championed by authors

like Annett (9) who concludes that morphine in the opium poppy is a useless end product of metabolism. The plant, having no mechanism for excreting an end product of such a complicated structure, stores it in the capsules where it can do no harm to its own metabolism. Chaze (10) has also endorsed the above view observing that in the tobacco leaves, nicotine is localized in the cell cavities of the epidermis and oozing through the hairs on the leaves is so much that the concentration of the alkaloid is notably diminished in the epidermal cells at the base of the hairs. In the Himalayan *Berberes*, deposits of alkaloids have been detected in the outermost cells of the bark and in the dead cellular elements of the plant, where the accumulation of the alkaloids is harmless to metabolic activities of the *Berberes*.

On the other hand, Coulter, Barnes and Cowles (11) have mentioned that alkaloids might not be necessary products of metabolism; for the very plants that produce alkaloids most abundantly may be grown under conditions which produce no alkaloids. Such cases have not yet been found for either the European or the Himalayan species of *Berberis* plants. Since the actual excretion by plants is very imperfect it might be mentioned that no proper casting of barks takes place in the case of the Himalayan *Berberes* and there is no provision for doing more than storing the alkaloids in some out-of-the-way places. In the course of varied chemical changes of protein breakdown, the alkaloids, accumulated, might be supposed to protect the *Berberes* from predatory animals.

MATERIAL AND MICROSCOPICAL STRUCTURE

The material—the roots, stems and leaves of the different species of *Berberis*—was collected during October and November, 1941, mostly form the outskirts of the town of Darjeeling, altitude, 7000 ft. The specimens of the three of the species, *B. lycium*, *B. vulgaris* and *B. Wallichiana* DC. var. *latifolia* were obtained from the Lloyd Botanical Gardens, Darjeeling. The material was softened by moistening it in a solution of formalin (5 cc.) in water (500 cc.). Hand sections of the stem, root and leaves were cut and examined under the microscope. Iodine (5 parts) in potassium iodide (10 parts) solution in water (100 parts) was used for staining the sections for a study of the distribution of alkaloids in different tissues, and borax-carmine or saffranine and aniline green were used to study the microscopical structures of the cells. The major alkaloid of the *Berberes* is colored yellow and as such its deposition can be viewed under the microscope, if the section is fairly thick and if the accumulation of the alkaloids is fairly large in the cells.

Transverse Section of a Fibrous Root of B. nepalensis Spreng.—A freshly cut hand section of a fibrous root showed that the outer cells of the cortical tissues contained a fair concentration of alkaloids. Central cells were comparatively free from alkaloids. Small groups of cells, many isolated cells of the cortex and those on the inner side of the phellogen showed a greater accumulation of alkaloids. The alkaloids were also distributed uniformly in the walls and cavities of the cells where they remained dissolved in the sap. With the staining solution, iodine in potassium iodide solution, the pale yellow regions of the alkaloidal deposits took an orange-red color. For the complete secondary growth, the phloem was highly compressed and took no stain, indicating the absence of alkaloids in the phloem. Large vessels of the xylem were free, whereas smaller vessels were full of starch grains. Certain walls near the pith were stained deep orange-red, indicating a greater accumulation of alkaloids.

Longitudinal Section of the Fibrous Root of B. nepalensis Spreng.—The longitudinal section revealed characteristics similar to those of the transverse section, both unstained and stained.

Transverse Section of the Main Root of B. nepalensis Spreng.—The structure of the main root was the same as that of the fibrous one. The alkaloidal deposit was found in tissues which were similar to those of the fibrous roots, with the difference that in the main root the concentration of the alkaloids in the wood and the cortical regions was very high.

Transverse and Longitudinal Sections of a Stem of B. nepalensis Spreng.—Both transverse and longitudinal sections of a stem, half an inch thick, showed thick deposits of alkaloids in the medullary rays, on the cell walls of the xylem and the hard basts of the phloem. The outermost regions of the xylem took an orange-red stain and its inner layers an orange-yellow. Cortical cells were stained brown. The cavities of the cells of the pith were noticed to contain alkaloids in granular forms and also in solution. The number of vascular bundles was 28.

Transverse Section of a Leaf of B. nepalensis Spreng.—The section of a full-sized mature leaflet through the mid-rib was stained deep yellow in the sclerenchyma in the mid-rib surrounding the vascular tissues. The sclerenchymatous tissues around the vascular bundles were stained orange-red and the layers of thick-walled hypodermal cell were pale brown. The cells of the mesophyll were free. The alkaloids were present in traces in the leaves. The number of vascular bundles was 3.

Transverse Section of a Root of B. insignis Hook. f.—On treatment with iodine in potassium iodide solution, the xylem and the medullary rays of the main root were stained orange-red. The thinner parts of the section took a light orange staining. The cells of the medullary rays contained some starch grains. Secondary growth was complete and the pith had been replaced by xylem plate.

Transverse Section of a Young Slender Root of B. insignis Hook. f.—The wood and the xylem plates took orange-yellow stain. The hard bast contained groups of orange-yellow cells and the bark some

isolated range-yellow cells. The phloem was free from alkaloids.

Transverse Section of a Stem of B. insignis Hook. f.—The section revealed that on the outside was the epidermis, and next was the cortex showing the parenchyma with the sclerenchyma. The internal cork showed 3 to 6 layers of rectangular cells. After them were a few layers of cortical cells. There were 20 vascular bundles, in close contact with one another radially. There was a large pith. Each vascular bundle had a well-developed sheath of sclerenchyma. The medullary rays were very thin. Both the sclerenchyma of the bundle sheath and the isolated groups of sclerenchyma in the cortex took an orange-red stain. The wood elements were yellow, and the cork was stained brown because of the presence of suberin.

Transverse section of a Leaf of B. insignis Hook. f.—In the mid-rib the pericycle sheath sclerenchyma, which consisted of 7 to 8 layers of cells, had been stained orange-red with iodine in potassium iodide solution. The wood or the xylem of the vascular bundles, 4 in number, took an orange-yellow stain. The bundles of the veins passing through the mesophyll showed similar colorations. The phloem remained unaffected. The epidermis was covered with a thick layer of cuticle.

Transverse Section of a Root of B. umbellata Wall.—Isolated strands of sclerenchyma were noticed in the inner and the outer parts of the root bark and in the hard bast and in the phloem which had been included in the bark. These strips took an orange-red stain. The layers of cork were noticed both in the outer and inner parts of the cortex where alkaloid deposits had been detected. In fact, alkaloids were fairly distributed in all parts of the root bark. The thick-walled cells accompanying the medullary rays which lay across the phloem were deeply stained like those of the sclerenchyma. The wood elements took an orange-yellow stain and the smaller wood elements surrounding the large pitted vessels which contained starch grains were colored blue and the thin walls were stained orange-red.

Transverse Section of a Stem of B. umbellata Wall.—The section of a young stem showed furrows and ridges on the outside. The epidermis was of a single layer of cells with hairs. The hypodermis consisted of 2 to 3 layers of sclerenchymatous cells. When treated with a solution of iodine in potassium iodide solution, the section was stained orange-red, showing the presence of alkaloids. The cell walls of the cortical parenchyma were slightly stained while the wavy pericycle was deeply stained. The phloem was free from alkaloids. There were 16 vascular bundles arranged in a ring, separated radially by medullary rays which contained numerous starch grains. The wood, the sclerenchyma, the pith and the cortical parenchyma were deeply stained orange-yellow. The xylem did not contain any protoplasm-containing elements.

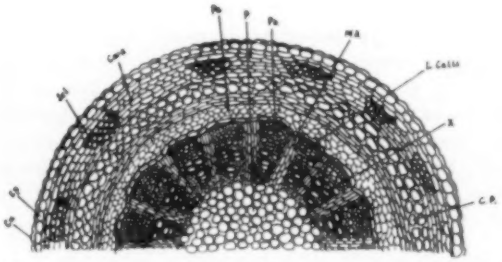

Fig. 2.—Transverse section of a stem of *B. umbellata*.

Cu, cuticle; *Ep*, epidermis; *Scl*, groups of sclerenchymatous cells; *Cork*, cork cells; *C.P.*, cortical parenchyma; *X*, xylem; *L.Cells*, lignified chain of cells; *M.R.*, medullary rays; *Pe*, pericycle; *P*, pith; *Ph*, phloem.

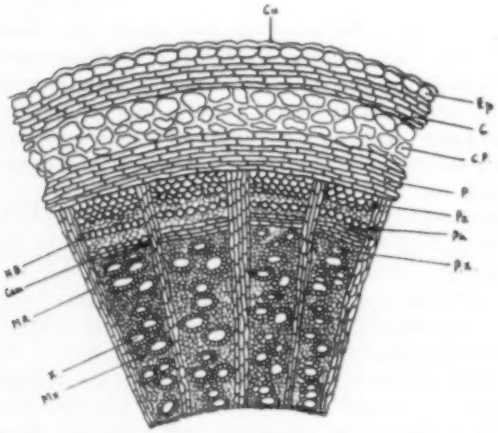

Fig. 1.—Transverse section of the root of *B. umbellata*.

Ep, epidermis; *C*, cork cells; *C.P.*, cortical parenchyma; *P*, phellogen; *Pe*, sclerenchymatous pericycle; *Ph*, secondary phloem; *H.B.* sclerenchymatous patches of cells; *Cam*, cambium; *Px*, protoxylem; *X*, xylem; *Mx*, metaxylem; *M R.*, medullary rays; *Cu*, cuticle.

Transverse Section of a Stem Bark with Lenticels of B. umbellata Wall.—Deposits of alkaloids had been detected both in dead cortical cells and cork cells.

Transverse Section of a Leaf of B. umbellata Wall.—When stained the xylem and the associated sclerenchyma in each bundle were turned deep reddish brown. The phloem in each bundle remained unchanged. The sclerenchymatous hypodermis was stained reddish brown and the sclerenchyma in the two edges of a leaf took an orange-red stain, showing alkaloidal deposits. The number of vascular bundles in the leaf was 4.

Transverse Section of a Root of B. Wallichiana DC.—The structure showed that on the outside was the bark, consisting of the dead epidermis, cork and cortical cells, which were deeply stained orange by iodine in potassium iodide solution. The smaller xylem vessels were stained deep orange showing alkaloidal deposits on the walls. The pith had disappeared. The cells of the medullary rays contained a very small quantity of starch.

Starch grains were also found in the tissues of the phloem. Groups of the pericyclic sclerenchyma had been stained orange-red.

Transverse Section of a Stem of B. Wallichiana DC.—In a young stem the vascular bundles which were arranged in a ring were found to be 16 in number. The xylem parts were separated radially from each other by narrow medullary rays. On treatment with iodine in potassium iodide solution, the medullary rays which contained numerous starch grains stained deep blue. The sheath in each bundle was unequally thickened and took and orange-red stain. The cells of the pith which retained cell contents were stained deep yellow. The phloem remained unaffected. It showed lignified chains of cells which were stained orange-red. The cork had formed near the endodermis, consisting of 3 to 4 layers of rectangular cells which were stained orange-red, showing the presence of alkaloids. The cortex was composed of 8 to 10 layers of cells. The epidermis was quite distinct and had a thin layer of cuticle. Alkaloidal deposits were most abundant in the sclerenchymatous heath of the xylem.

Transverse Section of a Leaf of B. Wallichiana DC.—The upper and the lower epidermis with cuticle were stained deep yellow. The small vascular bundles in the mid-rib (7 in number) and in the veins running between the palisade and spongy tissues had been stained orange. In the mid-rib, the pericyclic sclerenchyma consisting of 4 to 5 layers of cells surrounding the vascular bundles was stained deep orange. The xylem took a brown stain and the phloem remained unaffected.

Transverse Section of a Root of B. lycium Royle.—The elements of the wood in the central cylinder took an orange-red stain. The medullary rays were full of starch grains. Reserve starch was also noticed in the cells of the wood parenchyma. The pith had been replaced by a xylem plate and patches of sclerenchyma were noticed in the bark, which had been deeply stained.

Transverse Section of a Stem of B. lycium Royle.—There were 24 vascular bundles with 3 layers of sclerenchymatous sheaths. In each bundle, the smaller vessels were spiral, annular and strongly thickened. The epidermis contained multicellular hairs and the cortex consisted of 5 to 6 layers of cells. The section had been ruptured at the cambium. On treatment with the staining solution the bundle sheath everywhere was deeply stained orange-red, while the walls of the cortical cells were stained brown.

Transverse Section of a Leaf of B. lycium Royle.—A transverse section of the leaf through the mid-rib, when treated with the staining solution, showed that the sclerenchymatous cells, bast fibers and the scattered sclerenchymatous cells below the epidermis had turned orange-red, showing the presence of alkaloids. The number of vascular bundles was 7.

Transverse Section of a Root of B. vulgaris L.—The section of the specimen showed sceondary growth. The pith was replaced by xylem plate. The iodine stain revealed that the primary and secondary medullary rays were full of starch grains. The bark containing groups of lignified cells and the walls of all wood elements were stained deep orange-red showing a high concentration of alkaloids. The general cortex was brownish and in the secondary phloem, though the soft bast was unaffected, the solitary or groups of thick-walled bast fibers took an orange-red stain.

Transverse Section of a Stem of B. vulgaris L.—The number of vascular bundles was 18, arranged in a ring with a distinct and a fairly large pith at the center. The bundles were separated by narrow medullary rays and thickened strongly at the inner ends. Each of the bundles contained plenty of wood fibers, a few pitted vessels and some annular and spiral vessels. Outside the phloem there was a band of sclerenchyma, 3, 4 or 5 layers of cells in thickness. Outside this lay the phellogen and a periderm consisting of 3 to 4 layers of rectangular cells. Groups of bast fibers were noticed in the secondary phloem. It was found that on treatment with the iodine solution the wood elements in all bundles, and some of the layers of lignified cells in the hard bast took an orange-red coloration. While other groups of lignified cells in the hard bast were stained deep yellow, walls of the cortical cells appeared to be brown. The medullary rays and the pericyclic sclerenchyma and a few layers of cells on the inner side of the vascular bundles took a very deep blue stain, showing the presence of numerous starch grains. The pith was stained pale yellow. Wood fibers of the xylem as well as the bast fibers showed greater concentration of alkaloids.

Transverse Section of a Leaf of B. vulgaris L.—A section through the mid-rib of the leaf showed 3 vascular bundles. The sclerenchymatous tissues of the bundles of the mid-rib and of the veins and the wood elements were stained orange-red. The mesophyll lying between the upper and lower

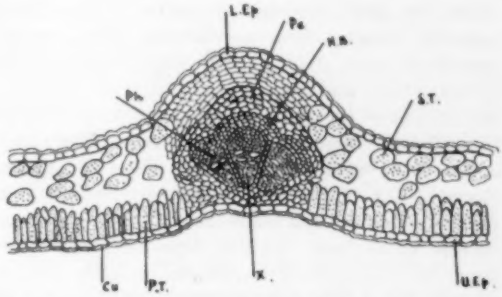

Fig. 3.—Transverse section of a leaf of *B. vulgaris* through its mid-rib.

L.Ep, lower epidermis; *U.Ep*, upper epidermis; *Pe*, sclerenchymatous pericycle; *H.B.*, hard bast; *Ph*, phloem; *X*, xylem; *Cu*, cuticle; *S.T.*, spongy tissue; *P.T.*, palisade tissue.

epidermis was differentiated into a single layer of palisade cells toward the upper epidermis and a spongy tissue toward the lower epidermis. These cells were free from alkaloids.

Transverse Section of a Root of B. Wallichiana DC. var. latifolia.—The xylem bundles were separated by narrow medullary rays which contained starch grains. The wood elements were stained reddish brown. The bark included groups of lignified cells which were also stained reddish brown. The xylem and the lignified cells of the bark were rich in alkaloids. The phloem was free from alkaloids.

Transverse Section of a Stem of B. Wallichiana DC. var. latifolia.—The section of a young stem was treated with the iodine solution. The cortex included a band of sclerenchyma of 3 to 4 layers and even 7 layers of lignified cells, which were stained orange-red. The vascular bundles, 22 in number, were arranged in a ring separated by narrow medullary rays which contained starch grains. Each bundle had a sheath of sclerenchyma which was stained deep orange-red. The wood elements also took an orange-red stain. There was a large pith, the small outer cells of which were stained orange-red. The soft bast was free from alkaloids.

Transverse Section of a Leaf of B. Wallichiana DC. var. latifolia.—A section through the mid-rib showed 3 vascular bundles surrounded by a sclerenchymatous pericycle, 3 layers in thickness. The sclerenchymatous sheath was stained deep orange-red. It was strongly thickened at the thin narrow ends where 3 bundles met. The hypoderma beneath the upper and lower epidermis, consisting of thick-walled cells, were stained yellow and the wood elements were stained orange-red.

Transverse Section of a Root of B. aristata DC.—On the outside was a single-layered epidermis, with cutinized cell walls. The formation of cork had started in the hypodermal region. The cork region was 8 to 10 layers in thickness and extended from hypodermis to pericycle. There were also isolated strands of sclerenchyma, 3 to 4 layers thick, in the hypodermal region. The wavy vascular bundles were continuous. The narrow medullary rays, 4 to 6 rows wide separated the xylem radially and ran from the center to the outermost region of the vascular bundles. At the center, the pith was replaced by a xylem plate. The xylem consisted of wood vessels, wood fibers and parenchyma. Crystalline brown deposits of alkaloids were observed on the walls, as well as in the wood vessels themselves. Yellow deposits of alkaloids were in the sclerenchymatous strands. When treated with iodine solution, the isolated strands of sclerenchyma, the hypodermis, wood fibers, wood vessels and the medullary rays had all turned deep orange-red showing the presence of alkaloids. The cutin in the outer walls of the epidermis had turned brown.

Transverse Section of a Stem of B. aristata DC.—The section of a young stem showed that on the outside was the epidermis. The parenchyma consisted of 3 to 4 layers of cells whose walls were not affected by the iodine solution. Next was a band of sclerenchyma consisting of 5 to 6 layers of thick-walled cells which took a deep yellow stain. The outer part of the phloem showed thick-walled cells which were not stained. The vascular bundles, 20 in number, were arranged in a ring. Each of the bundles was surrounded by a sheath of sclerenchyma which was stained deep orange-red showing concentraton of alkaloids. The wood vessels were all narrow and the walls were stained deep orange-red and the group of wood fibers was stained orange-red. There was a large pith of which the cells showed protoplasmic contents which had been stained yellow.

Transverse Section of a Leaf of B. aristata DC.—The section through the mid-rib showed 7 vascular bundles, surrounded by a many-layered sclerenchymatous pericycle, which was stained deep orange-red The wood fibers of the vascular bundles and the narrow ends were also similarly stained.

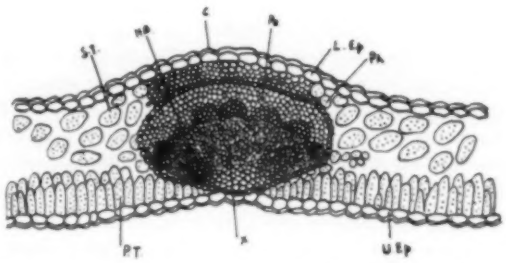

Fig. 4.—Transverse section of a leaf of *B. aristata* through its mid-rib, showing 7 vascular bundles.

L.Ep, lower epidermis; *U.Ep*, upper epidermis; *Pe*, sclerenchymatous pericycle; *H.B.*, hard bast; *Ph*, phloem; *X*, xylem; *Cu*, cuticle; *S.T.*, spongy tissue; *P.T.*, palisade tissue.

SUMMARY

Eight of the species of *Berberis* growing in higher altitudes of the Himalayas have been studied histologically with a view to understanding biological singificance of alkaloids present in them. It has been found by their histological distribution that the alkaloids are stored mainly in dead cells and also in other cells which take no part in metabolic activities and therefore the alkaloids are not drawn at all in matabolic changes. This observation, together with the accumulation of alkaloids of extremely bitter taste in the outer part (epidermis) of the stem bark, supports the views, gen-

erally held, that the alkaloids act as protective materials against attack of the plants by animals and that they are end products of metabolism rendered harmless to the plant and stored for the most part in special cells and regions where they are not readily reabsorbed into the active plant tissues.

The microscopical structures of the tissues of the roots, stems and leaves of the Himalayan species of *Berberis* have been described.

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The Assay of Ascorbic Acid*

By Edward J. Goett, F. Howard Hedger and Gordon O. Cragwall†

The majority of the proposed chemical methods for the analysis of ascorbic acid have been concerned primarily with the estimation of this vitamin in various natural substances (1–10). The first evidence in the literature of a critical interest in the possible shortcomings of the official United States Pharmacopœia assay of commercial ascorbic acid is that given by W. Bandaruk (11). Bandaruk notes the poor reproducibility of the iodine titration of ascorbic acid and proposes the use of an iodate solution in the presence of strong hydrochloric acid in place of the official iodine solution.

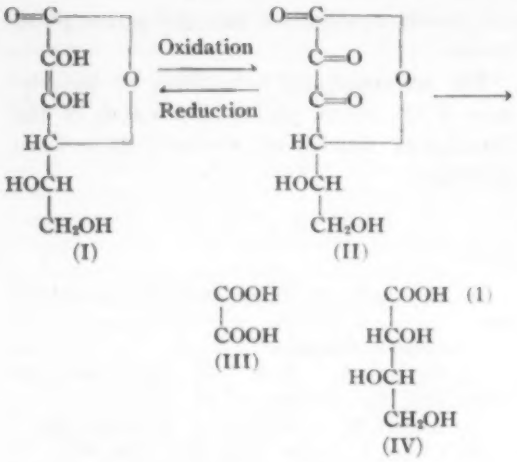
It has been the experience in these laboratories that more than the ordinary care must be exercised in the maintenance of temperature and dilution conditions in order to obtain reproducible assays of ascorbic acid using the iodine titration method. The authors have noted particularly that, even after repeated crystallizations of ascorbic acid samples, it was impossible to obtain an assay of greater than 99.3% with iodine solutions. A critical survey and investigation of the practical methods of ascorbic acid analysis has led to the conclusions that (*a*) the U. S. P. ascorbic acid assay, employing iodine in the presence of potassium iodide, does not give an accurate estimate of the purity of ascorbic acid due to adverse mass action effects of hydriodic acid, and (*b*) of all the methods investigated, the iodate-iodine monochloride method gives the only accurate determination of this purity.

* Received September 10, 1942, from the laboratories of Chas. Pfizer and Co., Inc., Brooklyn, N.Y.

† The authors wish to acknowledge the assistance of Mr. W. H. Staebner in the preparation of this paper, and the many helpful suggestions of Mr. T. Grenfell and Mr. D. Manley of these laboratories, whose past experience in ascorbic acid analysis and oxidation procedures contributed a great deal to the development of this work.

THEORY

The most reliable chemical methods for the assay of ascorbic acid are based on its oxidation-reduction characteristics. The chief oxidation-reduction reactions affecting these analytical procedures may be represented in Eq. 1 as follows:



The primary oxidation product of ascorbic acid (I) may be represented as the diketo compound, dehydroascorbic acid (II). This primary oxidation is called the "reversible" oxidation. The secondary reaction products, resulting from an "irreversible" oxidation are oxalic acid (III) and threonic acid (IV). This secondary oxidation is also termed a "decomposition" oxidation. The aim of the analytical oxidation procedures is to complete the primary oxidation without bringing about any oxidation of the second type.

The decomposition oxidation is effected by comparatively strong oxidizing agents such as permanganate or dichromate. Oxidation solely in the primary stage, namely, to dehydroascorbic acid, may be accomplished by mild oxidizing agents such as iodine and bromine. It has been shown experimentally that the reverse reaction, namely the reduction of dehydroascorbic acid back to ascorbic acid, may also be effected by very weak agents, even by hydriodic acid (12). Such a system would be expected to give rise to a very mobile equilibrium. Evidently the reaction would only proceed to completion when one of the reaction products was removed from the reaction system.

The official assay of commercial ascorbic acid is that adopted in the Second Supplement to the Eleventh Revision of the United States Pharmacopœia (13). The method is based on the reversible oxidation of ascorbic acid by an iodine-iodide titration mixture in a weak acid medium. The stoichiometric conditions at the end point may be represented in Eq. 2 as follows:

$$C_6H_8O_6 + I_2 \rightleftharpoons C_6H_6O_6 + 2HI \qquad (2)$$

In this simple iodine titration no attempt is made to remove the hydriodic acid from the reaction system. The end point is detected by the appearance of a blue coloration in the starch indicator imparted by the free iodine.

Hirst and Zilva (12) reveal that it is possible to produce ascorbic acid from dehydroascorbic acid by evaporation of just such a titration mixture as results under the above procedure. When this was attempted, they noted the evolution of iodine from the reaction mass. It is obvious that the evaporation of the reaction mixture led to the increased concentration of hydriodic acid, consequent shifting of the equilibrium (Eq. 2) to the left, and finally the complete reduction of dehydroascorbic acid to ascorbic acid. In preparing the iodine titration mixture, it is necessary to include between 4% and 5% of potassium iodide in order to insure the stability of the mixture. This naturally leads to an increased concentration of hydriodic acid in the reaction mixture, since in the acid medium the following reaction (Eq. 3) takes place:

$$KI + H^+ \rightleftharpoons HI + K^+ \qquad (3)$$

It would appear then, at least theoretically, that the standard procedure of the U. S. P. does not lead to a complete assay of ascorbic acid and results in a reaction mixture whose end point would be very sensitive to the temperature, dilution and acidity conditions in the mixture because of inherent mass action effects.

With the substitution of an iodate titration reagent for the U. S. P. iodine reagent, it is possible to reduce the concentration of potassium iodide in the reaction mixture. When iodate is substituted for iodine in the presence of potassium iodide (1), the reaction (Eqs. 4 and 2) proceeds as follows:

$$5KI + KIO_3 + 3H_2SO_4 \rightarrow 3I_2 + 3K_2SO_4 + 3H_2O \qquad (4)$$

$$C_6H_8O_6 + I_2 \rightleftharpoons C_6H_6O_6 + 2HI \qquad (2)$$

The final concentration of HI will depend upon the amount of KI originally added directly to the reaction mixture. If no KI

is added to the reaction mixture, the initial oxidation reaction (Eq. 5) will take place as follows:

$$KIO_3 + 3C_6H_8O_6 \rightarrow KI + 3C_6H_6O_6 + 3H_2O \quad (5)$$

As soon as a small amount of KI has been produced, the reaction probably proceeds as indicated in Eqs. 2 and 4. It will be seen that under these conditions five-sixths of the ascorbic acid oxidation will be due to the oxygen of the iodate and only one-sixth due to iodine with the consequent production of HI. It is obvious then that, by employing these modified iodate procedures, it is possible to control the amount of HI in the final reaction mixture, and thus make at least a qualitative study of the effect of HI concentrations on the final equilibrium.

The substitution of bromine and bromate solutions for the iodine and iodate solutions will lead to similar equilibriums, which would be expected to go more nearly to completion, due to the fact that bromine compounds are stronger oxidizing agents than the corresponding compounds of iodine, and, conversely, hydrobromic acid is a weaker reducing agent than hydriodic acid.

In order to avoid the incomplete reactions of the above-mentioned equilibriums, it has been pointed out previously that it would be necessary to remove one of the reaction products from the field of action. Hydriodic acid may be removed from the reaction mixture by employing the method of Andrews (14), in which iodate is used as the oxidizing agent in the presence of strong hydrochloric acid. In this method of assay the HI formed in the oxidation of ascorbic acid is removed by oxidation to ICl. The over-all reaction (Eq. 6) that takes place is as follows:

$$2C_6H_8O_6 + KIO_3 + 2HCl \rightarrow KCl + 2C_6H_6O_6 + ICl + H_2O \quad (6)$$

By employing this procedure it should be possible to obtain a complete quantitative assay of ascorbic acid samples, provided the oxidation conditions are not too severe.

EXPERIMENTAL

The ascorbic acid used in this investigation was of U. S. P. quality with a m. p. of 189–190° C., $[\alpha]_D^{25}$ of +20.95° (c., 10% in H_2O), and acidity as ascorbic acid of 99.7%. It was freed of surface moisture by drying in an air oven for 1 hr. at 105° C.

Reagents Used.—An approximately 0.1 *N* thiosulfate solution was prepared by dissolving 26 Gm. of sodium thiosulfate in 1 L. of distilled water. This solution was standardized against resublimed iodine of predetermined 99.88% purity. Frequent restandardization of the thiosulfate was employed throughout the investigation. All of the oxidizing solutions were standardized against this thiosulfate in the usual manner in acid solution in order to correlate the assays indicated by each oxidizing agent.

All oxidizing solutions were made up to approximately tenth normal strength, the iodine, iodate and bromide-bromate solutions being prepared according to U. S. P. XI, while the bromine solution was prepared in a manner analogous to the iodine solution by substituting KBr for KI. In addition, a special iodine solution was prepared by dissolving equal parts of iodine and potassium iodide in water. This solution was employed in a qualitative study of the effect of hydriodic acid concentration on the completion of the reaction in Eq. 2.

Results.—(1) Iodine Procedure: The standard procedure of U. S. P. XI was followed.

Run No.	Assay, %
10	99.13
11	99.12
12	99.10

Following the same procedure, a special iodine solution was employed which contained only one-third of the potassium iodide contained in the U. S. P. solution. This solution was not stable enough to warrant its use in standard analytical practice. Varying amounts of KI were added in the titrations to investigate the effect of the HI concentration mentioned above.

Run No.	Gm. KI Added	Assay, %
13	0.0	99.64
14	0.0	99.60
15	0.0	99.59
16	0.8	99.00
17	1.6	99.43
18	2.4	98.10

With the substitution of iodate for iodine solution in the U. S. P. procedure, the following results were obtained:

Run No.	Gm. KI Added	Assay, %
19	0.0	99.83
20	0.0	99.81
21	0.5	99.78
22	0.5	99.78
23	0.5	99.79
24	1.0	99.70
25	2.5	99.53
26	4.0	99.13

(2) Bromine Procedure: The method employed here is similar to that outlined by Schulek and co-workers (10) for their bromate-bromide procedure except that bromine solution was substituted for bromate solution and methyl orange was used

instead of *p*-ethoxychrysoidine hydrochloride indicator.

Run No.	Assay, %
7	99.83
8	99.85
9	99.82

(3) Bromate-Bromide Procedure: Schulek's (10) method was used with methyl orange indicator.

Run No.	Assay, %	Run No.	Assay, %
1	99.85	4	99.8
2	99.87	5	99.88
3	99.86	6	99.85

The end point in this procedure, while sharp, is very sluggish. This characteristic is also noted by Kolthoff (15).

(4) Iodate-Iodine Monochloride Procedure: The procedure employed in this titration is similar to that developed by Andrews (14). The details follow:

Weigh exactly 0.5000 Gm. of ascorbic acid on a watch glass and transfer carefully to a 500-cc. glass-stoppered flask. Dissolve the acid in 15 cc. of water and add 65 cc. of concentrated HCl. Cool the mixture to 18° C. Add 5 cc. chloroform, then introduce as rapidly as possible all but a few tenths of a cubic centimeter of the required KIO_3. Cool the reaction mixture again to 18° C. Titrate dropwise with vigorous shaking to the disappearance of the iodine color in the chloroform layer.

$$\text{Per cent ascorbic acid} = \frac{\text{cc. titer} \times \text{molarity of } KIO_3 \times 35.2}{\text{weight of sample}}$$

In the investigation of temperature effects, the same procedure was followed with an alteration of temperature conditions only. Unless otherwise noted, the end point in each case was stable at 20° C. for a period of at least eight hours.

Run No.	Assay, %
27	99.96
28	99.98
29	99.94
30	99.94

In the following runs the temperature was allowed to rise to 30° C.:

Run No.	Assay, %
31	99.87
32	100.21
33	100.80

In Runs 31 to 33, inclusive, it was not possible to obtain a stable end point. In Runs 27 through 31, the presence of oxalic acid in the final mixture could not be detected, while in Runs 32 and 33 a very definite test for oxalic acid was obtained.

In an investigation of the effect of hydrochloric acid concentration the following results were obtained:

Run No.	Final HCl Concn. Gm./100 Cc.	Assay, %
34	10	99.78
35	8	99.62
36	5	99.65

Attempts to estimate the ascorbic acid content of natural fruit juices by this procedure resulted in failure, since the presence of finely divided solid material formed an emulsion in the chloroform layer and prevented the observation of the end point. (Even if this could be avoided, the presence of reducing sugars, which would affect the iodate, would undoubtedly prevent the application of this method to the juices.)

CONCLUSIONS

Runs 13 to 26, inclusive, definitely establish the fact that the potassium iodide concentration greatly influences the equilibrium conditions in either the iodine or iodate-iodine procedures. While an exact quantitative relationship between the iodide concentration and the indicated ascorbic acid assay was not established, these determinations show that the greater the iodide concentration, the lower the indicated assay. In Runs 19, 20 and 21, titration with iodate gave an average assay of 99.80%, while an identical titration with an added 4 Gm. of potassium iodide in Run 26 reduced the assay to 99.13%. Runs 13, 14 and 15 show that with a specially prepared iodine solution which is low in KI, it is possible to obtain an ascorbic acid titration as high as 99.64%. With this special solution, addition of increasing amounts of KI to the reaction mixture reduced the assay to as low as 98.10% in Run 18. These results indicate that the present U. S. P. procedure for the assay of ascorbic acid is not reliable and is subject to adverse mass action effects which, even with extreme measures, cannot be completely eliminated.

Determinations 1 to 12, 19 to 23 and 27 to 30 clearly establish the difference in assay of ascorbic acid as indicated by the various methods of titration. It will be noticed that the iodate-iodine monochloride procedure (Runs 27 to 30) indicates the highest assay with the bromate (Runs 1 to 3), bromine (Runs 7 to 9), iodate-iodide (Runs 19 to 23) and iodine (Runs 10 to 12) following in decreasing ascorbic acid "purity." This substantiates the theoretical considerations discussed previously and indicates that the iodate-iodine monochloride procedure is the only one which completely oxidizes ascorbic acid to dehydroascorbic acid.

Runs 34 and 36 reveal that in the iodate-iodine monochloride procedure a final concentration of hydrochloric acid below 12% fails to give a complete assay of ascorbic acid. This confirms the observations of Andrews (14) and Jamieson (16) in their discussions of this procedure applied to other reducing substances. As they explain, the incomplete assay is probably due to the premature hydrolysis of iodine monochloride.

Runs 31 to 33 show that at temperatures as low as 30° C. the iodate-iodine monochloride procedure will cause some irreversible oxidation of the ascorbic acid. Failure to detect oxalic acid in Runs 27 to 30 indicates that the iodate procedure effects only the primary reversible oxidation of ascorbic acid when the reaction mixture is held below 20° C. The assays in these runs show that the oxidation of ascorbic acid in this stage is complete and obviously unaffected by mass action conditions.

SUMMARY

The investigation has shown that the present U. S. P. ascorbic acid assay is unreliable and subject to unfavorable reversibility, which prevents the complete oxidation of ascorbic acid. It has further established that of all the investigated methods under the proper temperature and acidity conditions the iodate-iodine monochloride procedure gives the only accurate assay of ascorbic acid. In the light of these results it appears that the latter method should be recommended in place of the iodine method in the assay of commercial ascorbic acid.

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Relationship of Chemical Structure to Germicidal Activity of a Series of Quaternary Ammonium Salts*†

By A. L. Rawlins, L. A. Sweet and D. A. Joslyn

Studies of the lower members of the homologous series of straight-chain alcohols (1) and alkyl phenols (2) have established that the germicidal activity increases and water solubility decreases with increase in the molecular weight. Attempts to extend this generalization to the higher homologues have led to contradictory results, because they are no longer water soluble in germicidal concentrations. In contrast, quaternary ammonium salts, even though of high molecular weight, usually are remarkably water soluble. This property appeared to offer an unusual opportunity to determine whether germicidal activity increases continuously in a homologous series, or whether a maximum is reached. Thus, without the limiting factor of water insolubility, it might be possible to discover a compound with phenomenal germicidal activity. Unfortunately, a maximum chain length was encountered beyond which the germicidal activity fell to very low levels. This corresponds closely with the findings of Miller (3) and his co-workers on the action of detergents on the metabolism of bacteria. They found that lauryl, myristyl and cetyl alkyl sulfates and sulfoacetates exert the maximum inhibition in those homologous series.

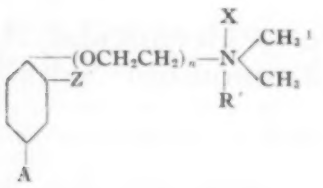
* Received August 6, 1942, from the Research Laboratories; Parke, Davis and Company; Detroit, Mich.

† Presented before the Medicinal Section of the American Chemical Society at the meeting in St. Louis in April, 1941.

Compounds of nitrogen have so many physiological applications that it is surprising that their germicidal possibilities were so long neglected. This evidently occurred because early investigators failed to hit upon the more promising types of compounds. In 1916 Jacobs (4) and co-workers reported the germicidal activities of derivatives of hexamethylenetriamine, but the real possibilities of this type of germicide were neglected until Domagk's (5) report in 1935 on long-chain quaternary ammonium salts. More recently several groups of American workers have reported studies in this field. Leffler (6) investigated a series of *N*-dodecyl heterocyclic amines and concluded that, in general, these are less germicidal than dodecyldimethylbenzylammonium chloride. Shelton (7) studied over 100 compounds and selected the alkyl pyridinium salts as offering most interest. In that series the optimum germicidal activity was found with cetylpyridinium chloride.

EXPERIMENTAL

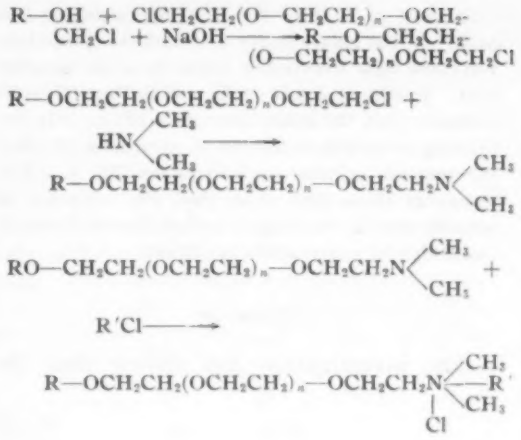
We have studied a group of quaternary ammonium compounds of the general type,[1]



in which A is alkyl, aryl, aralkyl or cycloalkyl; Z is halogen, alkyl, aryl or cycloalkyl; R′ is hydrogen, methyl or substituted methyl; X is chloride or other anion, and *n* is 2 or 3.

Many of these compounds were prepared by Bruson and co-workers (8). In general, the method of preparation was as follows: The appropriate phenol was reacted with an α,ω-dihalo polyalkylene ether using an alkaline condensing agent. The product thus obtained is reacted with dimethylamine and the resulting tertiary amine is converted to the quaternary ammonium salt by reaction with an alkyl or aralkyl halide.

[1] We wish to thank Dr. H. A. Bruson and Dr. D. H. Powers, Röhm & Haas Company, Philadelphia, Pa., for making available many of the compounds for this study.

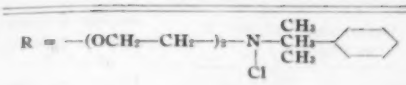
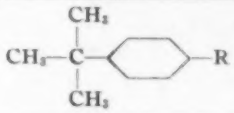
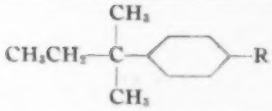
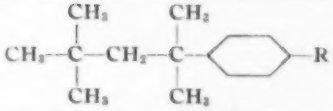
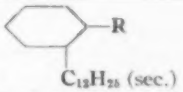
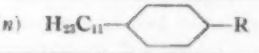


Most of the compounds studied were white crystalline solids, readily soluble in water in nearly all proportions, and gave clear, colorless and odorless solutions of *p*H slightly below 7. These solutions foam readily and have low surface tensions. The germicidal tests were run on these aqueous solutions using the Shippen modification of the F. D. A. technique. The tests were made on *Eb. typhosa* and *Staph. aureus* to find the maximum dilution which killed at 20° C. in 5 min. These were chosen as the most important test organisms representative of both gram-negative and gram-positive bacteria, respectively. For convenience in summarizing our studies, we have grouped the compounds according to structural characteristics to show the effect of variation in structure on germicidal action and surface tension.

Because of the greater ease of preparation of the diethoxy compounds, considerable attention was given to this series which is shown in Table I. A definite peak of activity is reached with *p-tert*-octylphenoxyethoxyethyldimethylbenzylammonium chloride even though longer chain compounds retain water solubility. Thus the *p*-(*n*-undecyl)-phenoxy derivative is not active even at 1:100 dilution. It will be noted that substituting a methyl group in the *ortho* position of the *para* substituted phenyl group reduces the germicidal effectiveness and yet the *o*-methyl-*p*-dodecyl compound is ten times more active against *Staph. aureus* than is the *p*-undecyl compound. The *o*-methyl-*p*-cetyl- derivative, on the other hand, is ineffective against both *Eb. typhosa* and *Staph. aureus* at 1 to 100 dilution. Aromatic, aralkyl or cycloalkyl substitution of the phenyl group gives uniformly low germicidal activity. The octyl substituted phenyl compounds are shown to be the most effective and among these the diisobutyl grouping (1,1,3,3-tetramethylbutyl) is the one of choice.

In Table II is shown the effect on germicidal action of changing the diethoxy to a triethoxy group. A direct comparison may be seen in the *tert*-octyl substituted phenyl group compounds, the triethoxy derivative showing much lower activity.

TABLE I

R = 	Dilution Germicidal at 20° C. in 5 Min.		Surface Tension of 0.1% Solution at 25° C., Dynes per Cm.
	Eb. typhosa	*Staph. aureus*	
	1,200	1,000	40.7
	3,000	2,500	45.8
	12,000	25,000	36.0
	900	900	30.3
(*n*) 	<100	100	32.0
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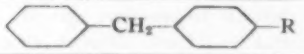
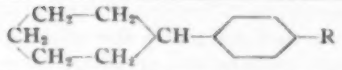
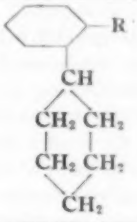
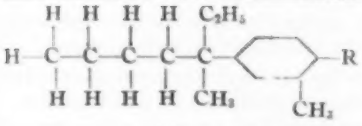
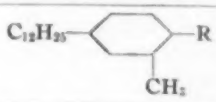
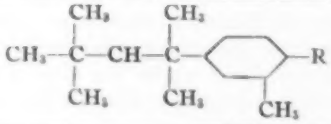
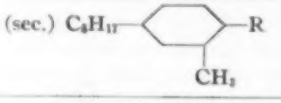
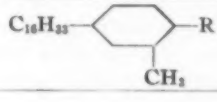
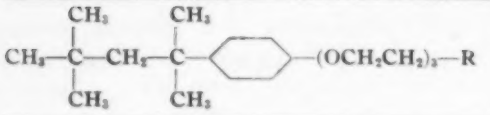
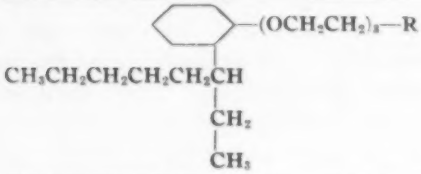
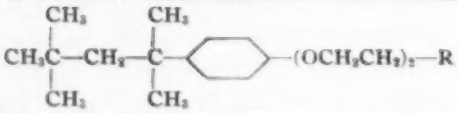
	2,800	2,800	52.7
	2,200	4,500	50.2
	2,200	4,500	52.6
	5,500	20,000	38.3
	100	1,000	31.0
	8,000	20,000	35.9
(sec.) 	3,500	18,000	28.9
	<100	<100	35.1

TABLE II

	Dilution Germicidal at 20° C. in 5 Min.		Surface Tension of 0.1% Solution at 25° C., Dynes per Cm.
	Eb. typhosa	*Staph. aureus*	
	8,000	10,000	34.4
	10,000	18,000	30.9
	12,000	25,000	36.0

In Table III is shown the effect of altering other groups attached to the nitrogen. Substitution of chlorine in the benzyl group lowers the germicidal activity against *Eb. typhosa* but not against *Staph. aureus*, except with the *o*-methyl-*p-tert*-octylphenoxy group. It has previously been shown that substituting a methyl group in this position lowers activity, with a further lowering resulting form substituting a chlorine in the *para* position of the benzyl group.

It is interesting to note that the tertiary amine, *p-tert*-octylphenoxyethoxyethyldimethylamine hydrochloride, shows only slight activity. Yet, when methyl is substituted for hydrogen to give the quaternary ammonium compound, the germicidal effectiveness increases threefold. If, instead of adding another methyl group, a benzyl group is added, a more than twenty-five fold increase occurs. Addition of a cinnamyl group increased the activity only slightly more than methyl, in contrast to the observation by Leffler (6) that it was superior to benzyl in enhancing the activity of their series of compounds.

Kuhn and Westphal (10) have reported remarkably high germicidal values for the ethobromide of *n*-dodecylbenzotriazole. For comparison we prepared the corresponding *p-tert*-octylphenoxyethoxyethyl derivative but found that in this instance it conferred less activity than the *n*-dodecyl radical. Attention is called, however, to the figures in Table IV which we obtained for Kuhn's compound.[2]

[2] This compound was prepared by the method of Kuhn and Westphal who reported a m. p. of 27° C, for the product as it crystallized from the reaction mixture. We found that this low melting material could be crystallized from dioxane, and after two crystallizations a m. p. of 85–87° C. was found.

	Found	Theory
Anal. C (av.)	60.73	60.76
H (av.)	8.15	8.35
N (av.)	10.61	10.60

While he reported an effective dilution of 1:615,000 against the organism loosely designated "*Staph.*," in our studies it showed a maximum effective dilution of only 1:800 against *Staph. aureus* in five minutes at 20° C.

It is often difficult to compare directly the results of germicidal tests from different laboratories. This is due partly to differences in the virulence of the organisms used and partly also to the selection of different temperatures or killing times as the criterion of activity. In order to determine the relative germicidal activity of different types of quaternary ammonium compounds, we have selected for comparison several of those which are considered the most active of their particular group and obtained the results shown in Table IV.

As a result of these studies *p-tert*-octylphenoxyethoxydimethylbenzylammonium chloride was selected for further investigation of its possible value as a general germicide. The results of those studies will be reported later in THIS JOURNAL.

Table V gives the results of studies of this product using the method of McCrea (9) which affords a very rigid test of fungicidal activity. It is effective against the three pathogenic fungi studied and ineffective against the two nonpathogenic fungi in the dilutions used.

The high bacteriostatic action of basic dyes suggested that bacteriostatic properties should also be found with quaternary ammonium compounds. This was found to be the case, *p-tert*-octylphenoxyethoxyethyldimethylbenzylammonium chloride exerting a bacteriostatic effect on *Staph. aureus* for one week at a dilution of 1 to 500,000.

Only a very limited correlation was found between surface tension and germicidal activity. The values given in Tables I to IV show that the more effective germicides have, in general, low surface tensions. On the other hand, some of the less effective ones are equally surface-active.

TABLE III

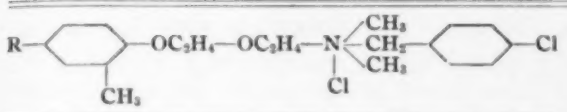
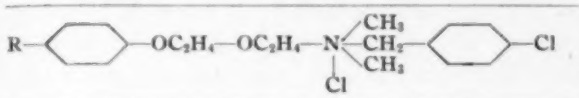
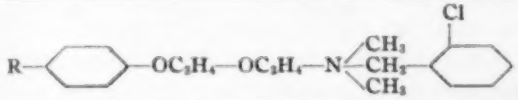
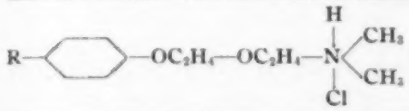
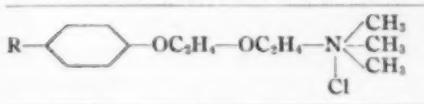
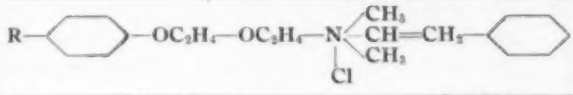
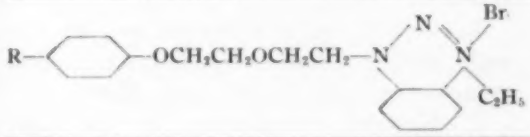
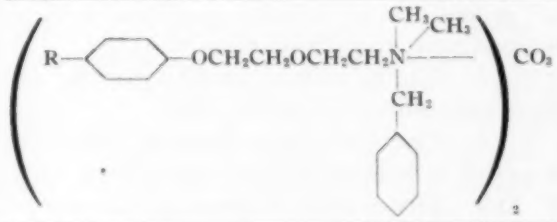
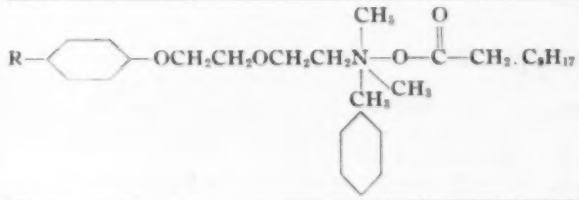
	Dilution Germicidal at 20° C. in 5 Min.		Surface Tension of 0.1% Solution at 25° C., Dynes per Cm.
	Eb. typhosa	*Staph. aureus*	
	4,000	2,000	30.6
	3,000	25,000	33.8
	8,000	25,000	34.7
	900	900	35.7
	2,500	3,000	40.4
	1,500	6,000	34.2
	4,000	2,500	45.9
	12,000	25,000	43.0
	9,000	15,000	27.1

TABLE IV

	Dilution Germicidal at 20° C. in 5 Min.		Surface Tension of 0.1% Solution at 25° C., Dynes per Cm.
	Eb. typhosa	*Staph. aureus*	
(*t*) C_8H_{17}[a]—$OCH_2CH_2OCH_2CH_2$—$N(CH_3)(CH_3)(CH_2)$—C_6H_5, Cl	12,000	25,000	36.0
(*n*) $C_{16}H_{33}$—$N(C_6H_5)$, Cl	4,000	9,000	41.7
(*n*) C_nH_{2n+1}—$N(CH_3)(CH_3)(CH_3)$—C_6H_5, Cl[b]	15,000	10,000	40.2
(*n*) $C_{13}H_{25}$—N=C_6H_4=N—Br, C_2H_5	10,000	8,000	29.5

[a] (t) C_8H_{17}—is 1,1,3,3-tetromethylbutyl referred to in text as tert. octyl.
[b] (C_nH_{2n+1}) represents a mixture of alkyl radicals ranging from C_8H_{17} to $C_{18}H_{37}$.

TABLE V

(*t*) C_8H_{17}—C_6H_4—$OC_2H_4OC_2H_4$—$N(CH_3)(CH_3)(CH_2)$—C_6H_5, Cl

Fungicidal Values—Method of McCrea (9)

	Dilution Fungicidal at 30° C., 5 Min.
Trichophyton interdigitale (No. 205)...	>1-1000
Epidermophyton rubens (No. 571).....	>1-1000
Monilia albicans (No. 545).....	>1-1000
Microsporum felineum (No. 556).....	<1- 100
Aspergillus niger (No. 228).....	<1- 100

CONCLUSIONS

As a result of this and published work, we have concluded that the general configuration of the molecule of the quaternary ammonium salt is as important as the exact chemical nature of its substituents.

1. The cation should preferably contain one long alkyl or alkylarpoly-(oxyalkyl) chain (possiblyl alkylaroalkyl would do just as well), one short aralkyl group and two lower alkyl groups.

2. The total length of the long chain should be 12–16 atoms. In approximating the chain length benzene rings were counted as 4 atoms. Any appreciable increase or decrease seriously interferes with germicidal activity.

3. Closed ring substituents on the aromatic nucleus are definitely inferior to alkyl groups in enhancing germicidal activity.

4. Halogen substitution in the aryl groups does not increase germicidal activity and may actually decrease it.

5. The anion may be derived from any simple mineral or organic acid.

6. One compound, *p-tert*-octylphenoxyethoxyethyldimethylbenzylammonium chloride, offers promise as a general germicide.

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(3) Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exptl. Med.*, 73 (1941), 249.

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(6) Leffler and Volwiler, presented at Sept. meeting, Am. Chem. Soc., Boston, 1939.

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(8) Bruson, H. A., U. S. Patents 2,115,250, 2,170,111, 2,229,024, 2,132,674, 2,098,203, 2,097,441.

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The Effects of Pentamethylentetrazol (Metrazol) on the Stomach*

By Hans Mautner† and I. Jacques Yetwin‡

It is well known that pentamethylenetetrazol (metrazol) stimulates the central nervous system including the cortex, medulla and spinal cord. Gellhorn (1) and Wang (2) also believe a stimulation of the hypothalamic region influences the autonomic nervous system. Only one experimental investigation has been carried out on the influence of the drug upon the gastrointestinal tract itself, that of Camp (3), who in subconvulsive doses found that metrazol increases the amount of gastric juice to a slight extent and in most instances causes a transient relaxation of the intestine which followed quite regularly, but did not occur after section of the splanchnic nerves.

Many clinical investigations on the effect of metrazol in convulsive and subconvulsive doses indicate the appearance of undesirable sequelae such as nausea and vomiting. Russell (4) found the vomiting could be prevented by giving a dose of atropine one hour prior to injection.

Stimulation of the adrenals is not the cause of the metrazol effect, since Haury and Gruber (5) showed that ligation of the entire adrenal gland effected no changes. Gellhorn (6) also suggests that metrazol exerts its influence on both the sympathetic and parasympathetic systems and has no direct connection with an increased epinephrine output.

Technique.—To analyze the effect of metrazol on the stomach we used a method which was successful in many other experiments: Koref and Mautner (7), Basch and Mautner (8) and Ebel and Mautner (9). Rats were kept without food or water for a period of 24 hrs. and then fed exactly 5.0 cc. of water through a rubber tube and syringe. A mouth-bit was used to prevent constriction of the tubing. Some of the rats received intramuscular injections of metrazol; others remained as controls. Both groups were killed by decapitation 20 or 30 min. after feeding. Most of the injected rats were killed 20 min. after injection. The stomach was ligated at the cardia and at the pylorus. It was then removed from the body and placed in a small funnel which rested on top of a graduated cylinder. The stomach was then cut several times with a pair of fine scissors and reduced to small pieces. The fluid dropping into the graduate was measured, and, when so desired, was removed for gastric analysis for free and combined hydrochloric acid according to the method suggested by Todd and Sanford (10).

The dose of metrazol decided upon was 0.25 cc. of a 1% aqueous solution per 100 Gm. of body weight. This dose and this concentration were relatively high but the rats were always destroyed long before a maximum effect could be observed. The majority of rats showed no convulsions, but some exhibited slight twitchings or convulsive seizures of short duration.

EXPERIMENTAL RESULTS

The Effect of Metrazol on Volume of Gastric Contents.—The following table is a résumé of our protocols to show rats injected with metrazol retain a larger amount of fluid in the stomach than those not injected. The volume of gastric juice is increased threefold with metrazol. After 15, and after 30 min., the stomachs of the control animals were completely emptied; the intestines and cecum were full. The stomachs of the metrazol rats contained fluid; the duodenum and upper portions of the intestines were also full but the greater portion of the intestines was entirely empty. This indicates there were no spasms of the pyloric muscles as a result of the disturbances of the autonomic nervous system because the duodenum would then also be empty. Fluid increases may be the result of hindrance to absorption, hypersecretion of gastric juice, or immobility of stomach musculature. Hypersecretion would be associated with an increased vagus effect; immobility would be due to a preponderance of the sympathetic nervous system.

* Received June 22, 1942, from the laboratories of the Department of Pharmacology, School of Medicine, Middlesex University, Waltham, Mass.

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‡ Assistant Professor of Pharmacology and Parasitology.

TABLE I.—THE EFFECT OF METRAZOL ON STOMACH VOLUME

Controls			Metrazol		
Rat	Protocol	Cc. Recovered	Rat	Protocol	Cc. Recovered
1	11-19	0.5	2	11-19	4.5
6	11-23	0.0	3	11-20	2.0
12	1-20	0.0	4	11-20	4.0
16	1-20	0.4	5	11-20	2.5
24	3-25	1.0	7	11-23	1.0
25	3-25	0.5	8	11-23	1.0
51	3-27	2.0	9	11-23	1.5
52	3-27	0.0	10	11-23	2.0
55	5-8	0.0	11	11-23	1.5
62	6-11	0.5	14	1-20	2.2
66	6-16	2.0	15	1-20	2.0
73	7-14	1.0	26	3-25	3.0
74	7-14	3.0	27	3-25	3.0
75	7-14	1.0	28	3-25	3.5
76	7-14	0.0	30	3-25	4.0
			43	3-27	3.0
			44	3-27	2.0
			45	3-27	1.8
			46	3-27	3.0
			69	7-14	1.0
			70	7-14	3.0
			71	7-14	2.5
			72	7-14	3.0
Average volume		0.8			2.5

To determine if the volume of secretion was actually increased we fed another series of rats 5.0 cc. of a 1:20,000 solution of Acid fuchsin. This made it impossible to perform gastric analyses on the samples, but slight diminution in the degree of redness could be observed in the injected animals and leads us to believe there was a slight increase in the secretion.

The Effect of Adrenalectomy.—Incisions were made on either side of the spinal column of etherized rats. Loops of silk thread were used to tie off the adrenals which were then entirely removed. In the "pseudo-operation", the adrenals were exposed but not removed.

We found an increased volume of stomach contents in several rats after bilateral adrenalectomy. Since the color of the instilled Acid fuchsin was a deeper red in adrenalectomized rats than in the animals with a "pseudo-operation," we can draw no definite conclusions at the present writing. Adrenalectomy has some effect on the functions of the stomach, but just what that effect is requires further investigation.

The Effect of Metrazol on Stomach Acidity.—Several of the recovered specimens were analyzed for free and combined acid. The results are noted in the following table. On the whole, we found a very slight increase in total acid in the injected rats. There was proportionately a greater degree of free acid in the metrazol animals. The figures obtained were so small because of the great dilution of gastric contents after gavage.

The Effect of Metrazol and Atropine on Volume and Acidity.—A group of animals received the usual dose of metrazol and in addition received, at the same time, a subcutaneous injection of 0.3 cc. of a 1:1000 solution of Atropine sulfate per 100 Gm. of body weight. Very slight diminution in gastric juice and total acidity were found, as indicated in Table III.

TABLE II.—THE EFFECT OF METRAZOL ON GASTRIC ACIDITY

Controls		Cc. 0.1 *N* HCl		Metrazol		Cc. 0.1 *N* HCl	
Rat	Protocol	Free	Total	Rat	Protocol	Free	Total
51	3-27		0.003	26	3-25	...	0.005
73	7-14	0.0005	0.002	27	3-25	...	0.001
74	7-14	0.003	0.004	28	3-25	...	0.003
75	7-14	0.0005	0.001	30	3-25	...	0.006
				43	3-27	...	0.0005
				44	3-27	...	0.002
				45	3-27	...	0.001
				69	7-14	0.0005	0.002
				70	7-14	0.001	0.004
				71	7-14	0.0025	0.003
				72	7-14	0.0035	0.004
Average acidity		0.0013	0.0025			0.0019	0.0029

TABLE III.—THE EFFECT OF METRAZOL AND ATROPINE ON VOLUME AND ACIDITY

Rat	Protocol	Cc. Recovered	Cc. 0.1 *N* HCl (Total)
23	3–25	2.5	0.002
29	3–25	2.7	0.002
31	3–25	2.0	0.003
32	3–25	3.5	0.000
47	3–25	3.0	0.003
48	3–27	2.0	0.001
49	3–27	2.0	0.003
50	3–27	2.2	0.002
Average results		2.5	0.002

The Effect of Metrazol on Stomach Motility.—To determine the rapidity of movement through the stomach and at the same time eliminate the troublesome factor of absorption and secretion, a series of rats received gavages of liquid petrolatum (mineral oil). Some of these animals were injected with metrazol at the same time; others acted as controls. Table IV represents our findings in that series. After injection we invariably found the stomach and upper intestines strikingly filled. In the control rats, very little of the liquid petrolatum could be found in the stomach and autopsy revealed the lower intestinal tract filled practically to the cecum.

From these experiments it is evident that the increased volume of gastric juice after administration of metrazol is not due to an absorption factor and only in a very low degree to a stimulation of secretion, but chiefly to an immobilization of the stomach musculature.

A similar relaxation of the bronchial musculature after metrazol inoculation was described by Januschke (11, 12) who claims metrazol is an excellent preparation for the relaxation of smooth muscle.

TABLE IV.—THE EFFECT OF METRAZOL AFTER PETROLATUM GAVAGE

Controls			Metrazol		
Killed directly after feeding			Killed directly after injection		
Rat	Protocol	Cc. Recovered	Rat	Protocol	Cc. Recovered
83	7–14	1.5	79	7–14	3.5
84	7–14	1.5	30	7–14	3.5
85	7–14	2.0	31	7–14	3.0
			32	7–14	3.0
Killed 20 min. after feeding			Fed. Injected. Killed in 20 min.		
77	7–14	0.0	36	7–29	1.5
78	7–14	0.0	87	7–29	3.0
92	7–29	0.5	88	7–29	3.0
93	7–29	0.3			
			Fed. Injected. Killed in 60 min.		
			89	7–29	2.5
			90	7–29	2.0
			91	7–29	Stomach full

SUMMARY AND CONCLUSIONS

1. The effect of metrazol on the stomach of rats has been studied. Specifically, that effect was one of an increase in stomach fluid contents following injection of the drug.

2. This increase is the result of cessation of movement through the gastrointestinal tract, and possibly a slight hypersecretion.

3. The apparent immobilization of the stomach cannot be attributed to an increased output of epinephrine. It seems probable there is a central effect on the autonomic nervous system.

4. These results corroborate the findings of several investigators in that a stimulation of the hypothalamic region simultaneously regulates both sympathetic and parasympathetic divisions of the autonomic nervous system.

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An Investigation of *Viburnum Opulus**

By C. H. Costello and E. V. Lynn

The viburnums have long been used in medicine and have many times been examined in the laboratory without eliciting a satisfactory judgment as to therapeutic worth. The histological reports date back to 1728, the chemical to 1817, and the pharmacological to 1897, while clinical records started in early Indian medicine.

The conflicting statements that have been made in every phase of research on the viburnums are apparently explainable when one examines the records critically. In nearly every case previous to 1940 the material employed was not satisfactorily identified and, since commercial samples have continually been found to be grossly adulterated or completely substituted, we can assume that very few of the results reported up to recent years here any important significance.

We have found in the literature relatively few reports on the chemical and none on the pharmacological investigation of authentic bark of *Viburnum opulus* L. var. *americanum* (Miller) Aiton. Viehoever, Ewing and Clevenger (1), in 1918, found some evidence that the volatile acids are isovaleric and butyric and that the tannins give a green color with ferric salts. Clevenger and Ewing (2) determined in 1920 the amount of ash and ether extract, while Powers and Powers (3) isolated the amyrins. Gustafson (4) failed to find an alkaloid but described an unstable glucoside which was not analyzed. In the only extensive investigation, made by Heyl (5) in 1922, there were isolated a phytosterolin, a phytosterol, myricyl alcohol, tannin, sucrose, glucose, and a paraffin. A glucoside was indicated but no alkaloid, and several acids were partly identified, including acetic, valeric, caproic, caprylic, formic, oleic, linoleic, cerotic and palmitic. None of these reports show much basis for therapeutic use.

Having available a large amount of authentic material and access to considerable stands in the field, we have submitted the bark to an examination. The chemical work was done for the purpose of tracing the potent substances, with a view to isolation, and was not intended to be complete. Several fractions, obtained in a variety of ways, have shown definite action on the uterus *in vitro* and *in vivo;* the evidence of this will be reported in a later paper. The present relates only the chemical work, although the pharmacological results are noted.

EXPERIMENTAL

The material was collected in Michigan in 1940 and was identified by Dr. H. W. Youngken as the bark of *Viburnum opulus* L. var. *americanum* (Miller) Aiton.

Preliminary Analysis.—A proximate analysis of the dried bark gave the following results, expressed in percentage by weight: adhering wood, less than 5%; foreign organic matter, less than 2%; total ash, 7.22–8.03%; acid-insoluble ash, 0.49–0.62%; moisture by oven method, 8.89–8.98%, by toluene method, 8.97%; volatile ether extract, 1.82–1.95%; non-volatile, 25.95–29.41%; alcohol-soluble, 30.87–31.57%; water-soluble, 31.03%; benzin-soluble, 23.55%.

Successive extraction of a sample in a Soxhlet apparatus yielded residues in order as follows: benzin, 6.53%; ether, 2.62%; chloroform, 1.87%; ethyl acetate, 3.54%; alcohol, 7.37%. In each case the solvent was evaporated and the residue was dried at 100° C.

The Enzyme.—In order to ascertain whether an enzyme was present, 500 Gm. of the ground bark was extracted. The aqueous solution, prepared by

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maceration for 48 hrs. and filtration, was mixed with an equal volume of alcohol. The resulting precipitate was filtered, washed with alcohol, and dried over sulfuric acid, yielding 0.55 Gm. of dark brown residue. This, mixed with an equal weight of pure amygdalin and moistened with water, gave within 30 min. an odor of benzaldehyde, and the evolution persisted for several weeks. It would seem that the bark contains a beta-glycosidase and a reasonable assumption would be that it also contains a glycoside.

Alcoholic Extract.—For the purpose of a more complete examination, several large portions of the ground bark (about 1500 Gm. each) were exhausted with alcohol in a Soxhlet apparatus. The resulting dark brown liquid was freed from alcohol at room temperature by vacuum distillation to give about 40% of dark, viscid material. After buffering to a *p*H of 7.5, this was found to have considerable sedative action on the uterus.

The extract was diluted with water and distilled with steam over about 12 hrs. The distillate, amounting to about 6 L., was cloudy, had numerous oily globules floating on top, was acid to litmus, possessed an odor of old apples but not valeric, and had a pungent taste that gave a numbness of the tongue and lips persisting for several minutes. In some cases this oil congealed in the condenser to a wax-like solid but melted again when room temperature was reached. The oil, collected by means of ether, amounted to an average of 0.8% of the dried bark. During collection there was deposited from the ether solution a small amount of crystals which had a melting point of 113° C. but was not sulfur (5). The oil had a specific gravity at 20°/20° of 0.904, was greenish brown to reddish brown in color, possessed a strong, unpleasant odor, and was acid to litmus.

Volatile Oil.—The oil was also obtained by macerating the bark with water and then distilling with steam, the yield varying from 0.13 to 0.31%. It was similar in odor and appearance to that from the alcoholic extract and was found to be definitely active on the uterus and uterine strips. The specific gravity at 20°/20° was 0.9305 to 0.9356, the refractive index at 20° C. was 1.4283, and the optical activity was slightly levo. The acid value of 330.5 corresponds to 35.41% of acetic acid or 60.2% of valeric acid. The ester value of 147.1 corresponds to 33.6% of $C_{12}H_{20}O_2$.

After dissolving in ether, the oil was extracted completely by a solution of sodium carbonate. The aqueous layer, after washing with ether, was acidified, and the separated mixture of acids was collected by means of ether. This yielded a yellow to light brown liquid with a pleasant odor, amounting to 50.95 to 56.82% of the oil. It gave a strongly depressant action on the uterus and apparently is mostly responsible for this action in the oil. In order to discover something about its character, a part of the mixture was subjected to fractional precipitation with silver nitrate, and each of the seven fractions was analyzed for silver in the usual way.

Fraction	% Ag Found	% Ag Calculated for	
1	39.7	Capric	38.7
2	42.5	Nonoic	40.7
3	46.8	Caprylic	43.0
	47.3	Heptylic	45.6
	47.8	Caproic	48.4
4	51.9		
	52.3	Valeric	51.7
	50.3		
	51.3		
5	57.1	Butyric	55.3
	54.9		
	55.0		
	53.5		
6	57.6	Propionic	59.7
	58.8		
	57.8		
7	62.2	Acetic	64.6

Evidently the mixture contained several acids, varying from 2 to 10 carbon atoms.

The residual ethereal solution was extracted with diluted sodium hydroxide to give a small amount (1.18% of the oil) of dark brown, viscid material, which was pharmacologically active. It had a strong odor of creosote and gave several general reactions of phenols and a phenylurethane melting at 78° C. From the extract a few crystals were deposited with a melting point of 115° C.

From the ethereal solution, after removing phenols, sodium bisulfite removed 1.16% of viscid, light brown liquid with a fruity odor. It gave an iodoform reaction of methyl ketones but was inert physiologically.

The oil remaining in the ether, presumably containing mostly esters, with perhaps hydrocarbons and alcohols, was a cloudy, light brown liquid with a pleasant aroma. From it was obtained a phenylurethane melting at 77° C. Since it apparently had no action on the uterus, any further examination was postponed.

Oils from Other Species.—Grote and Colburn (6) found volatile acids in the bark of *Viburnum prunifolium*, which would indicate a volatile oil. From authentic root-bark of this species we have obtained by distillation 0.28 to 0.33% of light brown oil containing particles of colorless, wax-like crystals. It possessed a faint odor like vanilla but became somewhat valeric when exposed to the air. It was found to be active on uterine muscle.

From authentic bark of *Viburnum alnifolium* Marsh was obtained by distillation 0.2 to 0.4% of yellowish brown oil with a penetrating, musty odor, becoming valeric upon exposure. This oil, which was also found to be actively depressant to the uterine muscle, deposited a small amount of yellow crystals on standing.

The Resin.—The residue from distillation of the alcoholic extract contained a physiologically inert, brown resin amounting to 15.9% of the bark. From it petroleum benzin extracted more than 50% of a dark green semi-solid, and ether withdrew another

14.1% of green, viscous material. The residue yielded brown, amorphous powders to chloroform, ethyl acetate and alcohol in the order named, but was mostly insoluble.

The Aqueous Solution.—The filtrate from the resin, which might possibly contain glycosides, yielded only a small amount of material to ether, 0.12% of the bark, but amyl alcohol extracted 1.42% of a dark, reddish brown, amorphous solid. This was only slightly soluble in cold water, more soluble in hot, and was completely soluble when buffered to a *p*H of 7.5 with sodium bicarbonate. This solution gave a definite effect on the uterine muscle.

The liquid that remained after extraction with amyl alcohol was concentrated to a smaller volume and completely precipitated by lead subacetate. The filtrate, after being freed from excess lead, was examined pharmacologically and found to be quite active. The reddish brown precipitate was suspended in water and decomposed by hydrogen sulfide, the mixture was filtered and the filtrate from this was also definitely active.

One is justified in concluding that the bark must contain several ingredients that have a depressant effect upon the muscle of the uterus, at least one or two of these being found in the volatile oil.

SUMMARY

1. The bark was submitted to chemical separation in order to trace compounds that have depressant action on the uterus. Such substances were found in the volatile oil, the aqueous solution of the alcoholic extract, the extract of this by amyl alcohol, in the precipitate by lead subacetate, and in the filtrate from it.

2. A beta-glycosidase was found present in the bark.

3. The volatile oil was found to contain more than 50% of acids, apparently containing from 2 to 10 carbon atoms, as well as esters, phenols and aldehydes or ketones. Oils were also obtained from the barks of *Viburnum prunifolium* and of *Viburnum alnifolium*.

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An Analytical Study of Tincture of Ipecac*

By Frank E. DiGangi† and M. S. Trupp‡

A study of the National Formulary assay method for Tincture of Ipecac was undertaken because students had difficulty with the official procedure and seemed to be unable to obtain concordant results. The assay method was troublesome owing to the formation of emulsions. Incomplete extraction of the alkaloids was indicated by low results obtained with this procedure.

Most of the tinctures of the Pharmacopœia and National Formulary are prepared by extracting the crude drugs. Tincture of Ipecac is an exception, being manufactured from the fluidextract. Studies were made to produce a working formula for the preparation of this tincture from the crude drug, so that it will meet the present official standards.

P. A. Self (1) believes that the production of emulsions is solely responsible for the poor results obtained in assaying preparations of ipecac. The formation of emulsions may be attributed to the presence of mucilage, tannins, saponins, soaps formed by the action of the alkali on resins, fats or fatty acids in the drug, or to the presence of solid particles precipitated by the addition of alkali, acid or simply by dilution with water.

The Fourth Edition of the National Formulary first provided a method for the

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assay of Tincture of Ipecac. It was identical with the pharmacopœial method for the fluidextract. Sawdust was dropped as the adsorbent in the Fifth Edition and the concentrated tincture was directly extracted with ether after alkalinization. This procedure was retained in the Sixth Edition.

Ether should be retained as the preferred immiscible solvent. G. Frericks and N. de Fuentes Tapis (2) found that ether would extract the principal alkaloids, emetine and cephaeline, but would prevent extraction of the less important psychotrine, which is soluble in chloroform. Only peroxide-free ether should be used. Bye, Schoetzow, Harrisson and Seif (3) confirmed the conclusions of other workers that ether containing peroxides yielded erratic results.

In this investigation the accuracy and simplicity of the official method for the assay of Tincture of Ipecac was compared with other methods and an attempt made to devise a new procedure.

EXPERIMENTAL

A sample of U. S. P. XI Ipecac, packaged by J. L. Hopkins and Co., was assayed according to the official method and found to contain 2.52% of ether-soluble alkaloids. A fluidextract (4) and subsequently a tincture (5) were prepared from this lot of drug by the official methods. The tincture was found to contain 0.15% of ether-soluble alkaloids when the National Formulary assay method was employed. This result is below the standard requirements. It remains the same when the tincture is evaporated at either 60 or 100° C.

Other samples of this tincture were assayed using the method as outlined for the fluidextract in the Pharmacopœia, but with some modifications.

MODIFIED U. S. P. METHOD

A 50-cc. sample was concentrated on a water bath to a volume of 10 cc., adsorbed with shredded filter paper and evaporation continued to dryness. The procedure from this point on is essentially that of the Pharmacopœia with several exceptions. In the beginning, an aliquot portion of the ether solution was washed with distilled water and this in turn with fresh peroxide-free ether. The latter was then combined with the original ether solution and the aqueous washings rejected. Fiftieth-normal alkali was used for the residual titration in place of the customary tenth-normal solution.

A number of samples assayed 0.21% ether-soluble alkaloids using this modified pharmacopœial method. This result lies within the official requirements for the tincture.

At this point in the investigation, two lots of tincture were prepared directly from the crude drug in contrast to the tincture made from the fluidextract. Two methods were used to prepare the tincture from the crude drug.

Method A.—Macerate 100 Gm. of the drug, No. 60 powder, for 72 hrs. and percolate using a menstruum of 80% alcohol (6) until the drug is exhausted. Reduce the percolate to 500 cc. on a water bath, add 2 volumes of distilled water and allow to stand overnight, filter and evaporate the filtrate to 620 cc.; to this liquid add 7.5 cc. of hydrochloric acid and 250 cc. of alcohol. Mix well and filter. A portion of this was assayed using the modified U. S. P. method and the remainder diluted with a mixture of 3 volumes of diluted hydrochloric acid, 25 volumes of alcohol and 72 volumes of distilled water to make 100 cc. of the tincture contain 0.18 to 0.22% of ether-soluble alkaloids.

Method B.—Evaporate the percolate obtained as outlined above to 300 cc. To this add 7.5 cc. of hydrochloric acid, 250 cc. of alcohol and 320 cc. of water. Mix well and filter after allowing to stand overnight. Assay a portion and adjust to the desired strength as given under *Method A*.

The tinctures made by the above methods will be denoted hereafter as Tinctures *A* and *B*. They were assayed using the modified pharmacopœial procedure. They contained an average of 0.22% and 0.23% ether-soluble alkaloids. These tinctures were prepared in an attempt to improve upon the N. F. VI method. *Method A* is preferred to *B* since it is easier to control the alcohol content of the finished product. Tincture *A* has the same concentration of alcohol as the official fluidextract and tincture.

Palkin and Watkins (7) proposed a hand shake-out method using the acid filtrate of the sample for extraction. This procedure did not yield concordant results when collaborators used it. This led them to devise a new method (8) which, however, showed no improvement over the then official process. Palkin, Watkins and Murray (9) suggested a mechanical continuous extraction for the alkaloids, but this method was shown to be equaled by the hand shake-out method since the time was doubled as shown by Bye, Schoetzow, Harrisson and Seif (3).

The hand shake-out method appeared to be the ideal one to use. It was simple to carry out and involved only one shake-out with the immiscible solvent, ether. The time actually spent on the entire assay procedure was about 4 hrs. On investigating the procedure, the answer to why collaborators could not get concordant results was apparently in the preparation of the acid-filtrate. The method as outlined by Palkin and Watkins (7) was changed as follows:

Evaporate a mixture of 100 cc. tincture and 5 cc. normal sulfuric acid on a water bath to 40 cc., cool, add 30 cc. of water and transfer quantitatively to a 100-cc. volumetric flask. Add enough water to bring it up to volume. Mix well and filter, rejecting

the first 10 cc. of filtrate. Ten cubic centimeters of the filtrate is equivalent to 10 cc. of the tincture.

MODIFIED PALKIN-WATKINS METHOD

The method of assay for the tincture is simplified once the acid-filtrate is prepared. The following procedure was used: Take 50 cc. of the acid-filtrate prepared as directed, add 2 successive 10-cc. portions of peroxide-free ether and shake vigorously in a separatory funnel for 2 min. Return the ether washings to a second separatory funnel and wash with 5 cc. of acidulated water. Transfer the acid washings to the first separator and reject the ether washings. Alkalinize the combined acid solutions, carefully, with ammonia test solution and extract with successive small portions of peroxide-free ether until extraction is complete when tested with Mayer's reagent. Evaporate the combined, filtered, ether extractions on a steam bath to about 5 cc., add 10 cc. more of ether and again evaporate to 5 cc. Effect solution of the alkaloidal material by warming with 10 cc. of tenth-normal sulfuric acid. Cool the liquid and titrate with fiftieth-normal sodium hydroxide solution using methyl red as the indicator. Each cc. of tenth normal acid is equivalent to 0.024 Gm. ether-soluble alkaloids.

The above method is simple, quickly run and free from formation of emulsions. It is more accurate than the aliquot ether method of the U. S. P. XI. In using this process, Tinctures *A*, *B* and *C* were found to contain 0.256, 0.264 and 0.246% of ether-soluble alkaloids, respectively, in the order given.

Chloroform was also used as the immiscible solvent in the modified Palkin-Watkins procedure. This is the solvent preferred by the British Pharmacopœia in the assay of preparations containing alkaloids. There is no advantage in the use of this solvent over ether since high results are obtained by the extraction of psychotrine and the heavy emulsions formed are dispersed only with the greatest difficulty.

TABLE I.—COMPARISON OF ASSAY METHODS FOR TINCTURE OF IPECAC

Methods	Tincture *A*	Tincture *B*	Tincture *C*
N. F. VI			
At 60° C.	...	...	0.145[a]
At 100° C.	...	...	0.150
Modified U. S. P. XI			
At 60° C.	...	...	0.210
At 100° C.	0.226	0.232	0.210
Modified Palkin-Watkins			
Ether	0.254	0.250	0.243
Chloroform	0.256	0.264	0.246
British Pharmacopœia	...	0.226	...

[a] Average per cent.

The results in the table indicate that the N. F. assay for Tincture *C* is inaccurate. The other two tinctures were not examined using this procedure since the same factors causing low results would be present. The modified U. S. P. XI method gives reasonably higher results for the 3 tinctures. Palkin and Watkins' method, when modified, also gives higher results which conform more nearly to those that should be obtained. When chloroform is substituted for ether as the solvent, the results are slightly higher, probably owing to the fact that psychotrine is extracted in addition to emetine and cephaeline alkaloids. The British Pharmacopœial method gives results approximating those obtained with the modified U. S. P. procedure. The former is somewhat difficult to carry out and offers many opportunities for occlusion and loss of the alkaloidal components during the assay owing to the presence of alcohol-soluble substances. These materials precipitate out of solution in large, resinous clumps upon alkalinization with ammonia.

SUMMARY

The modified Palkin-Watkins method is simple, accurate and gives more complete extraction of the alkaloids than other procedures. Only one extraction with ether is required. Less error occurs using an acid-filtrate rather than an aliquot of the highly volatile ether solution as in the modified U. S. P. XI assay. Chloroform gives higher results than ether owing to the extraction of psychotrine in addition to emetine and cephaeline.

The modified U. S. P. XI procedure for the tincture shows a distinct improvement over the N. F. VI method, simply because all alcohol is removed and resinous and other interfering substances adhere to the paper adsorbent.

The British Pharmacopœial method presents certain difficulties and yields results similar to those of the modified U. S. P. and N. F. VI although slightly higher. Emulsions are easily formed since alcohol-soluble principles are present in the extractive.

The N. F. assay is troublesome and impractical. Lower results are obtained with it than with any of the other procedures.

Tincture of Ipecac should be prepared as all other tinctures of the U. S. P. and N. F. from the crude drug and not from the fluidextract. The method preferred is that outlined under Tincture *A*. One made in this manner showed no precipitation when it was allowed to stand for 6 months. The N. F. VI tincture shows distinct precipitation in a like period of time.

CONCLUSIONS

A simple, accurate method is presented which does not yield troublesome emulsions and gives concordant results for Tincture of Ipecac. This method is recommended and preferred to the present N. F. VI assay procedure.

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A Study of Hydrophile Ointment Bases*

By G. W. Johnston† and C. O. Lee‡

In a previous paper by the authors (1), the so-called "absorption bases" were discussed in detail. The word absorption is used to denote their hydrophile or water-holding properties, and not to describe their action when applied to the skin. However, claims are made for the absorption of many of these preparations, and much work has been done recently on the activity and penetration of this type of product.

These hydrophile bases have found a definite place in pharmacy and more particularly in cosmetology because of their special properties. In reviewing the cosmetic literature, where mention is most often made of these products, it was noted that while claims for absorption were frequently made, little experimental evidence was offered as proof. Recent work, however, seems to promise some foundation for these claims.

Gershenfeld and Miller (2) recommended the use of water-miscible vanishing cream as a base for 2% phenol ointment. Such a preparation gave the greatest bactericidal efficiency

Fischler (3) in a study of emulsion types of ointment found that water-in-oil emulsions had greater penetrating properties than pure fats and petrolatums. He mentioned particularly cholesterol and oxycholesterol and certain patented products as emulsifying agents.

Poethke (4) used aquaphor in an investigation, and also suggested soaps and lecithins for the preparation of emulsion type ointments. He stated that the nature of the medicinal agent had great effect on the penetration.

Amrhein (5) showed that vitamin D was absorbed from the skin, but found that the vehicle had little or no effect on absorption since the vitamin was absorbed from both vegetable and mineral, and absorption bases.

Kramer (6) discussed these compounds at length, and stated that the presence of cholesterol in skin fats had the effect of permitting the surface of the skin to be easily moistened with water, and to take up water more easily in the form of emulsions.

In a study of cholesterol in ointments made by Li and Kuever (7), the writers postulated that the effectiveness of such

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preparations depended upon the extent to which they diffuse and absorb, and also upon the potency of the major ingredient. In investigating the antiseptic potency of various bases with ammoniated mercury and phenylmercuric nitrate, they found that ointments made with the water-holding bases had a distinctly higher potency than those without water.

Wood (8) conducted a study of the antiseptic value of emulsions of acriflavine, and his results led him to conclude that emulsions of the oil-in-water type had far greater bacteriostatic activity than those of the water-in-oil type.

Gershenfeld and Brillhart (9) carried out an experiment on the effect of ointment bases on the bactericidal efficiency of antiseptic agents. In all instances the agents in water-holding bases had far greater effect than those in the older types.

An entirely different approach to the effectiveness of such preparations was developed by Eller and Wolff (10). They tested bases on the skin of albino rabbits, performing biopsies at intervals after application. On examining the stained sections, they found that animal fat penetrated farthest into the skin, vegetable fats next, and mineral fats least. In addition, liquid fats showed greater penetration than solid fats, and all showed optimum penetration between 4 and 6 hours.

Kuever and Burnside (11) found that ointment of mercuric nitrate and ointment of phenol made with water-holding bases had several times the antiseptic potency of the older products.

Gibson and co-workers (12), in studying the emulsion type bases, found distinct advantages with the water-containing bases, and further found that bases of the oil-in-water type were best from the standpoint of stability and antiseptic action.

Duemling (13) studied wetting agents and their effect on skin penetration, using methods similar to those of Eller and Wolff. He found that fats alone penetrated chiefly about the hair follicles to a depth just below the skin; however, fats in combination with wetting agents penetrated to the base of the hair follicles rapidly, soon penetrated to a depth of 4 mm. below the skin surface and then spread out through the fibro-fatty tissue. It is interesting to note that Duemling mentioned the limitations of such products in cases where, therapeutically, skin absorption is not desired.

Foley and Lee (14) made a study of antiseptics in various ointment bases. Their results indicated that in general antiseptics were more active in hydrophile bases, but that the nature of the medicament had a profound effect on this activity.

Due to the interest that hydrophile ointment bases have been receiving, it was felt worth while to attempt to produce such a base to obviate the use of commercial or patented compounds. Thus it is the aim of this paper to discuss the problem of preparing an absorption base comparable to the commercial products and, further, to show that such a base may be used in the preparation of many of the ointments of the United States Pharmacopœia and the National Formulary.

EXPERIMENTAL

The foregoing discussion of absorption or water-holding bases gives a clue perhaps to their function as media for medicinal ointments and cosmetics. In a previous paper their approximate composition was discussed, and we showed that while cholesterol, when added to the usual fat-wax bases, greatly increased their emulsifying power with water, the emulsions were not stable (1). From information at hand, it seems that the presence of cholesterol esters, in addition to cholesterol, are the agents needed to help stabilize the water-holding bases which we were seeking to prepare. The cholesterol esters were not available so it was necessary to synthesize them. The following fatty acid esters of cholesterol were prepared: formate, acetate, propionate, butyrate, valerate, capronate, caprylate, caprinate, laurate, myristate, palmitate, and stearate.

The methods used for preparing these esters, while differing in detail, were essentially those of refluxing the acids with cholesterol under an atmosphere of carbon dioxide at temperatures of about 200° C. for varying periods of time. Purification was effected by recrystallization from such solvents as methanol, ethanol, methyl ethyl ketone and ether (15, 16, 17).

Water-Holding Bases.—The twelve cholesterol esters, in varying percentages, were all tested for their emulsifying power by combining them with cholesterol and white petrolatum, then incorporating 3 to 8 times their weight of water to form an emulsion. As the result of numerous tests it was decided

that there were no advantages in having concentrations of cholesterol and cholesterol esters higher than 3% in white petrolatum. The following general formula was adopted for detailed study.

FORMULA

Cholesterol	3 Gm.
Cholesterol ester	3 Gm.
White petrolatum	94 Gm.

Procedure: Warm the petrolatum to 140–150° C., add the cholesterol and cholesterol ester and stir until solution is effected. Stir occasionally while cooling.

Twelve bases were made using the 12 esters. Each base was easily emulsified with water, and the amount of the latter taken up varied from 300 to 800%. The emulsions were all reasonably stable showing only slight separation after standing for periods of 2 to 6 months. However, upon being rubbed on the skin, water almost invariably separated out in noticeable drops.

This left us faced with the problem of finding an agent that would stabilize the emulsions to the extent of preventing the separation of water when applied to the skin. Various combinations of the esters in the base failed to prove satisfactory. In addition to these, combinations of cholesterol, cholesterol esters, higher alcohols, waxes and anhydrous lanolin with white petrolatum were prepared. Anhydrous lanolin proved to be the agent sought and the following formula is proposed:

FORMULA BASE 20

Cholesterol	3 Gm.
Cholesterol stearate	3 Gm.
Anhydrous lanolin	25 Gm.
White petrolatum	69 Gm.

Procedure: Heat the petrolatum and lanolin to about 150° C., add the cholesterol and cholesterol stearate and stir until solution is effected. Stir occasionally while cooling.

Cholesterol stearate was used, inasmuch as it proved to be the ester which was most satisfactory in all respects. However, several other esters could have been used. White petrolatum is named in the formula but the most satisfactory brand was Sono-Jell No. 9. This base easily takes up 300% of water. It will emulsify much more water than this, but it hardly seems necessary to have a base with more than 300% water present. The emulsion showed no separation of water when rubbed upon the skin. Its spreading qualities were quite satisfactory, in fact just as good as the emulsions made from 2 of the much used commercial bases.

The properties of viscosity and melting point of Base 20 also compared quite favorably with the commercial bases.

Application of Base 20.—In checking the recommended uses for several of the commercial absorption bases, it was found that in nearly every instance the cholesterol-containing bases were incompatible with alkalies or soaps, or, more generally, with any substance producing an oil-in-water emulsion (18).

The advertising literature sent out by the manufacturers of several of the commercial water-holding bases contains information as to their uses and incompatibilities.

The behavior of Base 20 has been shown, in part at least, by using it as the base for the official ointments. A well-known commercial base was used as the control.

The general procedure was as follows: The proper amounts of the base and water were formed into an emulsion cream. The medicinal ingredients were then levigated with a portion of the emulsion until smooth, and the remainder gradually incorporated. In the case of liquid ingredients simple admixture with the cream was all that was needed. Where solids and liquids were both present, they were mixed, then added to the cream as described above. Where ingredients were water-soluble, they were dissolved in the water, then the solution emulsified with the base.

Very satisfactory U. S. Pharmacopœia ointments were produced with the following: boric acid, tannic acid, rose water, chrysarobin, ammoniated mercury, yellow mercuric oxide, sulfur, and zinc oxide. Ointment of pine tar was too soft and the addition of 10% white wax was necessary. Ointments of belladonna and phenol were unsatisfactory.

Of the National Formulary ointments, the following proved to be satisfactory: compound benzoic acid, calamine, capsicum, mild mercurous chloride, mercuric nitrate, red mercuric oxide, ichthammol, coal tar, lead oleate, neocalamine, potassium iodide, scarlet red, mustard, alkaline sulfur, and zinc stearate. Ointments of camphor, stainless iodine, compound menthol, compound tar, compound resorcinol, stramonium, and compound sulfur were all unsatisfactory. Some were stable for a few days, while others either did not form emulsions at all or broke immediately.

In all instances of incompatibility, the control with the commercial base behaved in similar fashion, and with the satisfactory ointments, in only two cases were the controls stable longer than those with Base 20.

Stability of Absorption Base Ointments.—The ointments prepared with absorption bases as described were subjected to variable temperatures in order to determine their stability at summer heat and at lower temperatures. They were placed in an oven at 34° C. for 24- and 48-hr. periods. Separate samples were placed in a refrigerator for the same length of time at 10° C.

Of the 26 satisfactory ointments studied, only 3 were unstable at 34° C.: pine tar, compound benzoic acid and stainless iodine. Of the controls, only stainless iodine was unstable at 34° C. At 10° C. only 13 ointments, about 40%, were stable. The controls behaved identically.

The water content of these ointments was variable due to their variations in strength of medicinal

agents. In all instances 33 Gm. of base were used with sufficient active ingredients for a 100-Gm. formula of official strength ointment, and enough water added to make 100 Gm. of product. Thus water content varied from a maximum of 200% to a minimum of 50%. About 85% of the ointments showed no separation of water after 30 days, and the remainder were stable up to 3 weeks.

SUMMARY AND CONCLUSIONS

1. Esters of cholesterol and the normal fatty acids were prepared and investigated as to emulsifying power. It appears that mixtures of cholesterol and cholesterol esters have greater emulsifying power than cholesterol alone, and of the esters, cholesterol stearate appears to be the best.

2. A satisfactory mixture, Base 20, was prepared, using cholesterol stearate and cholesterol in anhydrous lanolin and white petrolatum. It produced good ointments with many official ointment ingredients and compared favorably with commercial bases.

3. Volatile materials and alcohols appear to be incompatible with absorption bases of this type.

4. Almost all of the ointments prepared with Base 20 were stable at temperatures of high summer heat, although over half were unstable when subjected to a temperature of 10° C.

5. Ointments prepared with hydrophile bases and containing up to 200% of water do not remain stable indefinitely, hence these bases are best suited to the extemporaneous preparation of small amounts of ointments.

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Chlorobutanol in Parenteral Solutions*

By Abraham Taub† and William H. Luckey‡

Aseptic conditions and techniques are prerequisite to the manufacture of ampul medications, but conditions beyond the control of the most careful operator necessitate the use of some form of sterilization and subsequent proof of sterility before such medications may be considered safe for use.

Of the four methods of sterilization of ampul solutions that are official in N. F. VII—steam under pressure, fractional sterilization at 100° C., fractional sterilization at 60–70° C., and ultrafiltration—it is admitted that the latter two do not insure sterility. The use of an added bactericidal or bacteriostatic agent is therefore recommended. Furthermore, multiple dose vials, because they are subject to contamination during use, require the addition of a preservative agent whether sterilized by any of the four official methods.

Since the first use of a preservative for parenteral solutions in 1884, many chemicals

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have been suggested and tried for this purpose, among them phenol, cresol, halogen substituted phenols, mercurials, chlorobutanol and esters of *p*-hydroxybenzoic acid. The ideal preservative has still to be found. Chlorobutanol has one advantage over the other preservatives, and that is its local anesthetic action at the site of the injection. This accounts for its present extensive use, particularly in multiple dose vials.

Farwell (1) first used chlorobutanol as a preservative for biologicals in 1919 and found it effective. According to Hamilton (2), it has a phenol coefficient of 1.2. It is completely bacteriostatic in a concentration of 0.8% and will destroy *B. typhosus* upon 2 min. exposure. Briggs and Callow (3), using 0.5% chlorobutanol, found it efficient for non-sporing organisms. When used as a preservative in 2% procaine hydrochloride solution at *p*H 5.2, it destroyed *B. typhosus* and *S. haemolyticus* in 3 hrs.

Owen, Martius and Brosius (4), in evaluating several preservatives, prepared bacterial suspensions in saturated chlorobutanol solutions. After being heated for 1 hr. at 55–60° C., these solutions proved sterile. No clumping of organisms occurred, as with phenol and cresol. Davis (5), using parenteral solutions heavily contaminated with *S. aureus*, found that it required a 1:150 concentration of chlorobutanol to destroy this organism in 24 hrs., compared to 1:100 sodium propyl *p*-hydroxybenzoate, 1:120 phenol, 1:300 tricresol and 1:2000 *p*-chloro-*m*-cresol in the same period of time.

Chlorobutanol is found in a wide variety of ampul medications, both oil and aqueous. The aqueous solutions have a fairly extended *p*H range, from 3 to 9; some are buffered; many are unbuffered. They are sterilized under different conditions and temperatures, usually determined by the stability of the active medicament. However, little attention seems to have been paid to the stability of the added chlorobutanol.

It is the purpose of the present research to determine the optimum conditions under which this preservative may be used in aqueous solutions without decomposition. Since the hydrolysis of chlorobutanol results in the formation of hydrochloric acid, the decomposition may be measured by a determination of the chloride ion concentration as well as by the shift in *p*H.

EXPERIMENTAL

The solutions tested all contained 0.5% chlorobutanol in water. No other medications were present. Two series of solutions were prepared—one, unbuffered, from *p*H 3 to 9 at increments of one *p*H unit, and the other, buffered with McIlvaine's citric acid-disodium phosphate buffer from *p*H 3 to 8, and with Palitzsch's borax-boric acid buffer for *p*H 9. The *p*H of each solution was determined with the glass electrode prior to filling and sealing. Kimble hard glass ampuls were employed. They were boiled for 5 min. in 0.1% hydrochloric acid, drained, washed twice with distilled water, dried and sterilized at 160° C.

Each series of ampuls was divided into four groups. One was maintained at 25° C.; one was sterilized in a water bath at 65° C. for 30 min. on 3 successive days; one at 100° C. in moist steam for 30 min. on 3 successive days; and one was auto-

TABLE I.—EFFECT OF STERILIZATION TEMPERATURE AND STORAGE ON *p*H OF 0.5% CHLOROBUTANOL SOLUTIONS IN AMPULS

*p*H When Manufactured	*p*H 24 Hrs. after Sterilization				*p*H One Month after Sterilization			
	25° C.	65° C.	100° C.	121.5° C.	25° C.	65° C.	100° C.	121.5° C.
			A. Unbuffered Series					
3	3.02	3.05	2.45	2.31	3.03	3.04	2.45	2.31
4	4.05	4.01	2.51	2.31	4.02	4.00	2.50	2.32
5	5.02	4.58	2.50	2.36	4.46	4.50	2.52	2.35
6	5.22	4.85	2.49	2.35	4.60	4.74	2.45	2.37
7	5.29	4.95	2.50	2.36	4.61	4.76	2.48	2.36
8	5.37	5.00	2.50	2.36	4.67	4.91	2.50	2.36
9	5.70	5.10	2.50	2.41	4.72	4.85	2.50	2.40
			B. Buffered Series					
3	2.96	2.96	2.90	2.81	2.94	2.94	2.90	2.80
4	3.99	3.95	3.87	3.85	3.98	3.90	3.80	3.83
5	5.03	4.91	4.75	4.65	4.96	4.90	4.70	4.60
6	6.02	5.97	5.38	5.27	5.96	5.98	5.35	5.25
7	7.00	6.92	6.35	6.34	6.90	6.91	6.31	6.31
8	7.80	7.59	6.79	6.87	7.44	7.46	6.75	6.80
9	8.70	8.41	7.75	8.00	8.24	8.17	7.71	7.66

claved at 15 lbs. (121.5° C.) for 20 min. Ampuls from each group were opened at the end of 24 hrs., and at the end of one month, and the *p*H and chloride ion concentration of each were determined. The latter was determined turbidimetrically, using a Fisher Electrophotometer.

The results are indicated in Table I and Table II.

TABLE II.—EFFECT OF STERILIZATION TEMPERATURE AND STORAGE ON DECOMPOSITION OF 0.5% CHLOROBUTANOL SOLUTIONS IN AMPULS

*p*H When Manufactured	Decomposition of Chlorobutanol 24 Hrs. after Sterilization, %				Decomposition of Chlorobutanol One Month after Sterilization, %			
	25° C.	65° C.	100° C.	121.5° C.	25° C.	65° C.	100° C.	121.5° C.
			A. Unbuffered Series					
3	0.01	0.01	3.5	5.6	0.07	0.07	4.0	5.6
4	0.01	0.01	3.5	5.9	0.06	0.08	4.1	5.9
5	0.03	0.03	3.6	5.9	0.11	0.13	4.5	6.0
6	0.03	0.08	3.5	5.8	0.11	0.13	4.4	6.1
7	0.03	0.07	3.5	6.0	0.15	0.16	4.3	6.1
8	0.03	0.29	3.9	6.1	0.14	0.37	4.4	6.2
9	0.31	0.50	3.8	6.4	0.37	0.54	4.5	6.4
			B. Buffered Series					
3	0.01	0.01	3.5	5.4	0.04	0.07	3.8	5.5
4	0.01	0.01	3.8	5.7	0.07	0.08	4.0	5.7
5	0.01	0.01	7.3	11.3	0.08	0.12	8.1	13.0
6	0.03	0.10	16.7	30.6	0.27	0.39	22.0	37.0
7	0.28	1.10	32.4	54.6	3.00	3.30	54.7	58.0
8	3.33	10.00	60.3	61.0	13.00	16.00	62.7	62.8
9	4.00	30.70	62.0	72.6	16.70	64.00	66.0	75.3

CONCLUSIONS

1. The *p*H values of ampuls of unbuffered 0.5% chlorobutanol solutions, when heated much above 65° C., decrease rapidly to a constant low value (2.40 ± 0.10), irrespective of the initial *p*H, between the range *p*H 3–9. At temperatures up to approximately 65° C., the *p*H remains stable if the initial *p*H is not above 4.

2. The *p*H values of ampuls of buffered 0.5% chlorobutanol solutions remain stable at temperatures up to approximately 65° C. for initial *p*H values not above 7. For higher temperatures, the decrease in *p*H is much less pronounced than for unbuffered solutions.

3. Unbuffered chlorobutanol solutions remain fairly stable (decomposition less than 0.2%) for initial *p*H values up to 7, and temperatures not above approximately 65° C. At *p*H values up to 9, the decomposition is only slightly higher (0.5%). At autoclave temperatures the decomposition is nearly 7%.

4. Buffered chlorobutanol solutions remain fairly stable (decomposition less than 0.4%) for initial *p*H values up to 6, and temperatures not above approximately 65° C. At higher *p*H values, and at higher temperatures, they undergo much greater decomposition than unbuffered solutions, exceeding 70% decomposition at autoclave temperatures.

Although these inferences apply only to simple aqueous chlorobutanol solutions, and the presence of active medicinal agents may produce variations from the results noted, it is obvious that chlorobutanol should not be used beyond the conditions indicated for stability.

It is possible that the range of usefulness of chlorobutanol in ampuls may be extended by the use of auxiliary compounds, *viz.*, salts of weak bases and strong acids, which have markedly lower *p*H values at higher temperature than at room temperature, and thus permit sterilization at temperatures above 65° C. without appreciable decomposition. Work along these lines is now in progress.

SUMMARY

The effects of sterilization temperature, storage, *p*H and buffers upon the stability of chlorobutanol used as a preservative in ampul solutions have been indicated.

Chlorobutanol may be used between *p*H 3–9, in unbuffered solutions at temperatures not above approximately 65° C. Under these conditions there will be no material

decomposition of the chlorobutanol, although there will be a drop in *p*H from the initial value if the latter exceeds *p*H 4.

In the presence of buffers, chlorobutanol may be used up to *p*H 6 at temperatures not above approximately 65° C.

Buffered solutions, though useful from the standpoint of chlorobutanol stability through a less extended range than the unbuffered solutions, have the advantage of maintaining the initial *p*H after sterilization.

The optimum conditions of use, involving constancy of *p*H and stability of chlorobutanol, are:

In buffered solutions: *p*H 3–6; temperature: up to 65° C.

In unbuffered solutions: *p*H 3–4; temperature: up to 65° C.

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Determination of Nicotinic Acid and Sodium Nicotinate*

By Lewis E. Harris† and Beatrice I. Duis‡

Several methods (1, 2, 3, 4, 5) are available for either the titrimetric or colorimetric determination of nicotinic acid and sodium nicotinate. The titrimetric methods are satisfactory for nicotinic acid but not for sodium nicotinate. The colorimetric methods have disadvantages in that they may not be sufficiently rapid and they require the use of more or less expensive visual or photoelectric colorimeters. This report describes a method for routine control assay of nicotinic acid or sodium nicotinate in certain pharmaceutical preparations, based on the precipitation of nicotinic acid as the cupric salt. The following is the recommended assay procedure.

EXPERIMENTAL

Accurately weigh or measure a sample representing 50 to 100 mg. of nicotinic acid. If the solution is relatively concentrated, it is best to weigh or measure a quantity into a volumetric flask, dilute with distilled water and take an aliquot portion. Dilute the sample to approximately 50 cc. and carefully adjust to *p*H 7.1 to 7.3 (electrometrically) with either a dilute solution of sodium hydroxide or hydrochloric acid, as may be required. Add 10 to 15 cc. of a 12.5% solution of cupric sulfate, slowly with stirring, and allow to stand for 10 min. Collect the precipitate in a weighed Pyrex crucible with fritted disk (Gooch crucible may be used) and wash thoroughly with distilled water. Wash precipitate with 10 cc. alcohol and 10 cc. ether and dry to constant weight (usually 30 min. is sufficient) in an oven at 110° C. The weight of cupric nicotinate so obtained when multiplied by 0.7996 gives the amount of nicotinic acid in the sample.

It is also possible to collect the cupric nicotinate precipitate in a Gooch crucible and ignite to constant weight at a dull red temperature. The weight of cupric oxide so obtained when multiplied by 3.092 gives the amount of nicotinic acid present in the sample.

RESULTS

The results of over 50 individual assays show this method to be satisfactory for routine control work. The following table shows typical assay results of sodium nicotinate solutions.

TABLE I.—SODIUM NICOTINATE SOLUTIONS

Sodium Nicotinate (as Nicotinic Acid) Reported in Mg./Cc.

Sample No.	Theory	Assay	% Deviation
9422	100.0	99.7	−0.3
9433	100.0	97.4	−2.6
9434	100.0	98.0	−2.0
9731	100.0	99.4	−0.6
10042	100.0	99.3	−0.7
10255	100.0	101.8	+1.8
9453	100.0	99.8	−0.2

Each sample was run in duplicate. Results of duplicate assays show a maximum variation of ±2.0%.

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DISCUSSION AND SUMMARY

This procedure for the assay of nicotinic acid and sodium nicotinate, based on the precipitation as cupric nicotinate and subsequent weighing, was found to give good recoveries from solutions or tablets. The cupric sulfate reagent does not form a precipitate with thiamin hydrochloride, riboflavin or calcium pantothenate. However, the presence of certain combinations and concentrations of these members of the vitamin B complex cause low results in the nicotinic acid assay. The exact cause for this is not yet known. On the other hand, the presence of rice bran extract, liver extract or, beef extract in the sample causes high results probably due to precipitation of various cupric proteinates.

Several attempts have been made to use the method for the determination of nicotinamide after hydrolyzing to nicotinic acid. To date, this has not been entirely satisfactory and experimental work in this connection is still in progress. At present it appears that this method will be suitable for nicotinamide if it is first carefully and thoroughly hydrolyzed with nitrous acid and then all ammonia removed.

In general, this method, in its present form, is best adapted to the routine control assay of solutions or tablets of nicotinic acid or sodium nicotinate which neither contain more than one other member of the vitamin B complex nor contain any protein material.

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Book Reviews

An Outline of Organic Nitrogen Compounds, by ED. F. DEGERING, Department of Chemistry, Purdue University, CARL BORDENCA, Department of Chemistry, Alabama Polytechnic Institute, and B. H. GWYNN, Westvaco Chlorine Products Co., and collaborators. First planographed edition (previous editions, 1938, 1940). Planographed by John S. Swift Co., Inc., Cincinnati, Ohio. 1942. 381 pp., 15 x 23 cm. Price, $6.00.

This book represents the collected notes used by the senior author in a graduate course covering this extensive field. It is an attempt "to present in a concise and yet intelligible form the essential chemistry of the organic nitrogen compounds," and the authors hope that "this volume will serve both as a study outline and as a guide for the research chemist." It is not claimed that the book is comprehensive.

The first 3 chapters are concerned with "General Concepts." Relative electronegativity is explained, the fixation of atmospheric nitrogen discussed, and the ammonia system of compounds outlined. The next 28 chapters deal with the syntheses, physical properties and chemical behavior of those compounds in which the nitrogen is attached to an aliphatic or aromatic nucleus. Types included are nitro and nitroso compounds, oximes, amines (including amino acids, polypeptides and proteins), diazo and diazonium compounds, azoxy compounds, derivatives of hydrazine, amides and nitriles and their derivatives. Chapters on organic nitrogen dyes, alkaloids, nitrogen ring compounds and the isomerization of organic nitrogen compounds complete the text. A table of atomic weights and excellent author and subject indexes are appended.

Throughout the book the concept of relative electronegativity is used to explain the structures and reactions of the compounds discussed. Reactions of the various types of compounds are tabulated wherever possible, and the book is marked by the frequent use of equations and structural formulas for the purpose of illustrating the discussions. The outline arrangement makes for ready reference to specific information. Of special interest to the pharmaceutical chemist are the chapters on amino acids, polypeptides, proteins and alkaloids which, though brief, should serve as useful sources of information.—E. L. CATALINE.